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Plasmid Purification

Noboru Sasagawa

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Abstract

Plasmid purification is a rather classical experiment, but the technique is still developing for time- and cost- saving. The critical principle is based on the alkaline lysis method, although the following steps have several variations. The needed purities and/or quantities of DNA depend on researches using isolated plasmids, meaning that more reasonable method can be selected in each experiment. For example, a non-alkaline-lysis method such as boiling method is still available. One of the important steps for purifying plasmid is a removal of RNA. Ribonuclease is usually used for removing RNA from plasmid sample. On the other hand, a kind of salts such as lithium and calcium functions to make RNA as a selective precipitate from DNA-RNA mixture. Based on these backgrounds, the technique to purify plasmid DNA has been discussed.

Keywords: plasmid DNA, chromosomal DNA, RNA, RNase, calcium, polyethylene glycol

1. Introduction

It is said that the word “plasmid” is first proposed by the Nobel Prize winner Joshua Lederberg [1, 2]. Plasmid is an extrachromosomal small circular deoxyribonucleic acid (DNA), which duplicates independently from chromosomal DNA. Although budding yeast and fission yeast can retain plasmid, the host of the plasmid is almost bacteria. This small circular DNA is widely used as DNA vector in molecular biology, biochemistry, biotechnology, cell biology, and so on. It means that plasmid purification/isolation is very fundamental experiment in these research fields, and that this experiment is achieved in almost every laboratories on almost every day.

2. A strategy for purifying plasmid from *Escherichia coli*

In biochemical aspects, to purify plasmid DNA from bacteria is to isolate only plasmid DNA from the mixture of biopolymers such as protein, ribonucleic acid (RNA), chromosomal DNA and plasmid DNA, by which bacteria cell is composed (**Figure 1**).

A chemical property of protein is totally different from nucleic acids; therefore, it is rather easy to separate nucleic acids and proteins. However, RNA and DNA are very similar molecules from each other. Among them, ribose in RNA is only distinguishable from deoxyribose in DNA by one hydroxyl group (-OH) at its structure. Furthermore, chromosomal DNA and plasmid DNA is both deoxyribonucleic acids that have the same chemical properties. Chromosomal DNA in almost all bacteria is circular, so is also plasmid. The distinguishable difference of them is only their size: plasmid DNA (~10 kilo base pairs) is much smaller than chromosomal DNA (4.6 million base pairs in *Escherichia coli*). Based on these properties, a special technique for purifying plasmid DNA among these biomolecules are required.

To purify plasmid DNA of high quantity, culture condition, or media for *E. coli* growth is also important [3].

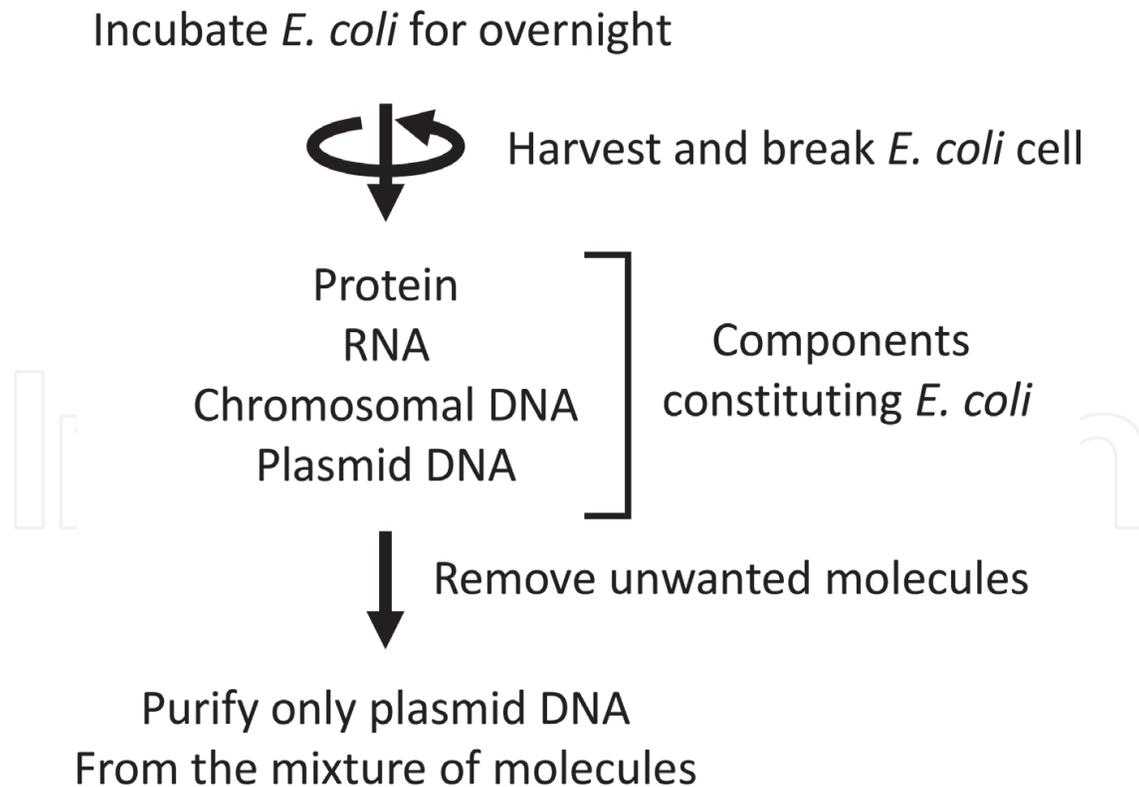


Figure 1. Strategy for purifying plasmid DNA from *E. coli*.

3. Very easy and simple way of plasmid preparation: boiling method

A very simple manipulation steps enable us to recover plasmid DNA from *Escherichia coli* (*E. coli*) (**Figure 2**) [4]. This experiment is called “Boiling method”. In this experiment, STET solution (100 mM sodium chloride, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5% Triton-X or Tween 20) is added to *E. coli* pellet and suspended well. And then, the sample is heated to 100°C for 1 minute and centrifuged. After centrifugation, plasmid DNA is recovered in the solution, whereas insoluble heat-denatured proteins make debris as pellet fraction. After separating plasmid DNA from debris and precipitating plasmid DNA by adding alcohol, the final plasmid sample is capable for the next experiment, such as cutting plasmid by restriction enzyme and/or modifying DNA by other enzymes.

Interestingly, the pellet is rather moisty and easily removed by using toothpick and piercing it. This feature contributes to an easy handling of the experiment: we can achieve this plasmid extraction in only one tube from the start point to the end of the experiment. Therefore, boiling method has very convenient for handling many samples at a time. The modified boiling method, in which a concentrated STET solution is directly added to LB medium in which *E. coli* is grown, is also reported [5]. This modified method does not require even harvesting step of the grown bacteria by centrifugation.

The major disadvantage of the boiling method is that RNA is not removed in the principle of the boiling method and that the chromosomal DNA of *E. coli* is not completely removed from plasmid DNA. Therefore, the purity of the finally isolated plasmid DNA is not so high.

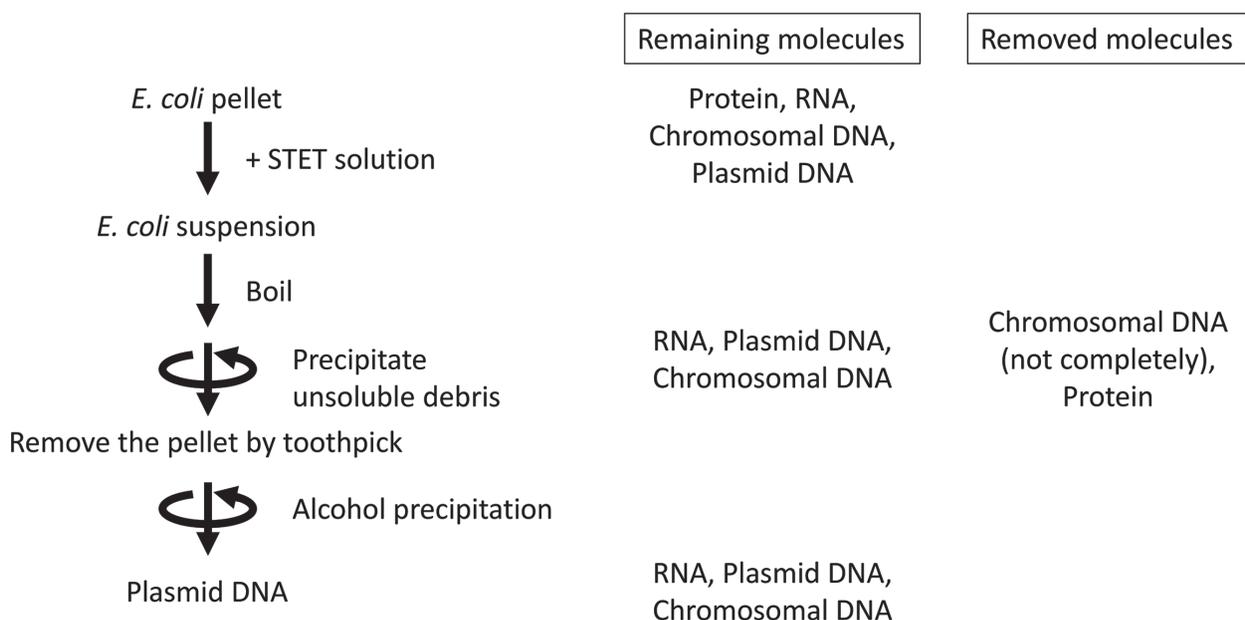


Figure 2. Scheme for purifying plasmid DNA by boiling method.

The boiling method is suitable for checking, if plasmid in *E. coli* transformant has an expected insert DNA (insert check), usually testing multiple samples at a time.

One substitution of the boiling method for insert check is colony polymerase chain reaction (PCR), directly adding *E. coli* colony to the PCR reaction mixture as a template. In colony PCR, *E. coli* cells are broken at the first 96°C step of PCR. Basically, a simple boiling of bacteria in the water is enough for collecting DNA from them, which has adequate quality to achieve PCR [6]. Colony PCR is much easier experiment than boiling method. However, once we isolate plasmid DNA even by rough purity of boiling method, we can further analyze the recovered plasmid by checking restriction enzyme patterns and so on. For example, restriction map is much informative result than if the fragment is amplified by colony PCR.

4. The definitive principle for plasmid isolation: denaturation of DNA double-strand by alkaline lysis

To purify plasmid from *E. coli*, there need each step for removing unnecessary molecules, such as protein, chromosomal DNA and RNA. For this purpose, alkaline denature of *E. coli* is the definitive technique for removing proteins and chromosomal DNA. This method was established in 1979 [7], but it is so sophisticated that almost all experiment for plasmid purification today is based on this technique (Figure 3).

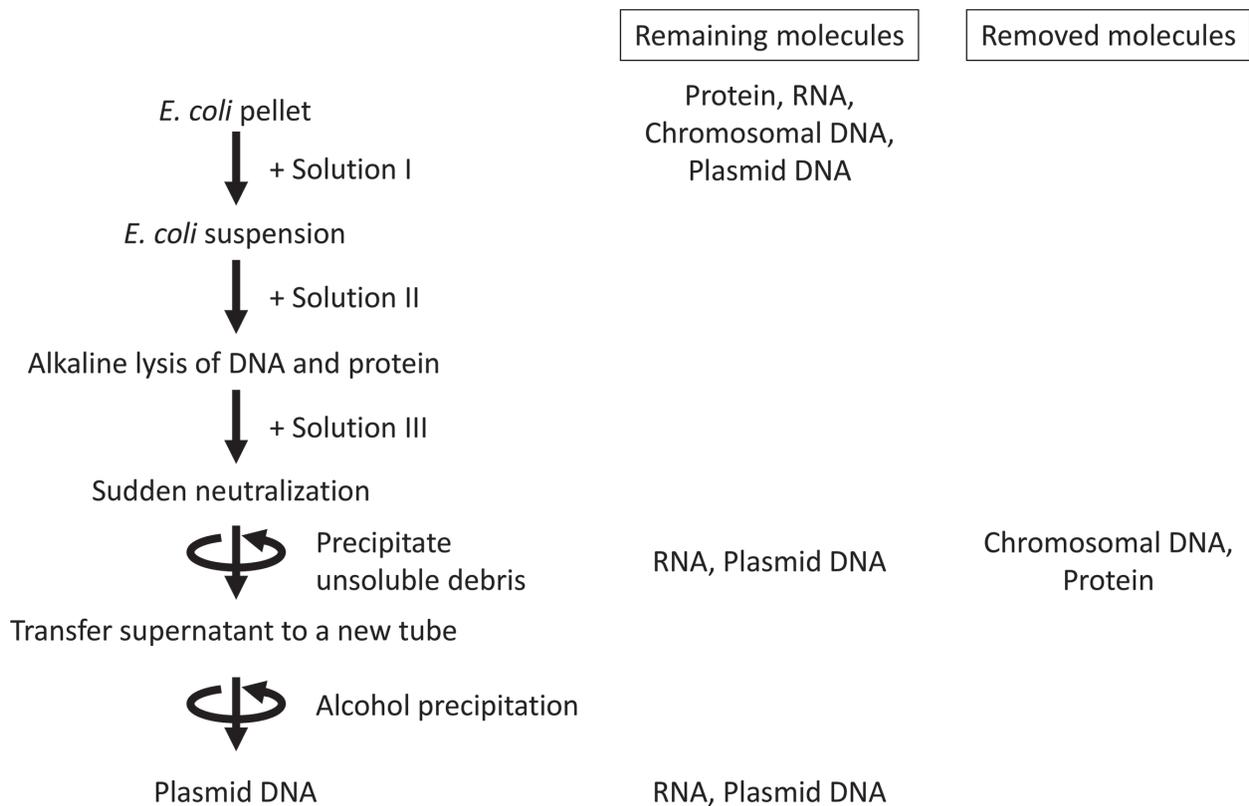


Figure 3. Scheme for alkaline lysis method.

The principle of the alkaline lysis method is a kind of magic. After suspending the *E. coli* in the solvent (solution I; 25 mM Tris/HCl (pH 8.0), 10 mM EDTA), an alkaline solution (solution II; 200 mM NaOH, 1% SDS) is added to the sample. In this condition, almost all proteins are denatured. DNA double-strand structure is also denatured to single-strand. However, even in such an extreme condition, supercoiled plasmid DNA remains its structure stable and not denatured. After 5 minutes, incubation of alkaline denature, high-salt buffer (solution III; 3 M Potassium Acetate, pH 5.5) is added for the purpose of a sudden change of pH in the solution. As a result, denatured protein and chromosomal DNA do not turn back to its own structure, causing these molecules insoluble. On the other hand, plasmid DNA remains soluble, thus centrifuge step easily separates the plasmid DNA from debris of proteins and chromosomal DNA. In the alkaline lysis method, each step is very simple and easy. All we have to do is only adding solution sequentially. Moreover, the function of each solution is only changing pH and salt concentration. However, these ingeniously planned three steps enable us to recover plasmid DNA, avoiding proteins and chromosomal DNA. The most notable point of this method is that we can isolate only plasmid DNA from plasmid/chromosomal DNA mixture; both are deoxyribonucleic acids and have the same chemical properties. No other method should successfully separate chromosomal DNA and plasmid DNA by such a simple step.

In early days, original protocol of alkaline lysis method used sodium acetate as a salt in solution III [7]. However, now potassium acetate is substituted for the major agent for solution III than sodium acetate. Potassium ion binds to dodecyl sulfate ion and forms potassium dodecyl sulfate (PDS). PDS is highly insoluble salt, which is made by adding solution III in the alkaline lysis sample. The PDS also plays a seed for insoluble debris, with which insoluble proteins and chromosomal DNA are co-precipitated. It is the great advantage that alkaline lysis method enables us to prevent protein and chromosomal DNA from plasmid at the same step.

One point we have to keep in mind is that supercoiled (closed circular) plasmid DNA is converted into nicked, relaxed (open circular) DNA by alkaline incubation. Thus, we have to keep the incubation time at solution II as in the instruction, and not to incubate the sample with solution II for a long time. Besides, RNA is not removed in a series of alkaline lysis method for plasmid purification (**Figure 3**). RNA is partially hydrolyzed by solution II but remains with the plasmid DNA at the final step. Therefore, a huge amount of RNA contamination is in the final plasmid DNA sample. Principally, only isolating plasmid DNA by alkaline lysis is inadequate for purifying high-quality plasmid DNA, and several schemes for further purification of the plasmid DNA, especially for removing RNA, should be needed.

Anyhow, alkaline lysis method has been the definitive way to initially purify the plasmid DNA.

5. RNA removal from plasmid sample

Neither alkaline lysis method nor boiling method does not isolate plasmid DNA from RNA mixture. An extra step for removing RNA is needed for further purification of plasmid DNA.

5.1. Ribonuclease (RNase)

Basically, to remove RNA (not to separate intact RNA) from DNA-RNA mixed solution is very easy: Only to add ribonuclease (RNase) to the solution enables us to completely digest RNA. Even when RNase is added to the solution I in the course of alkaline lysis method, RNA is completely digested in the finally corrected plasmid sample (see **Figure 3**). It sounds very strange that RNase in the solution I digest RNA, because the function of solution I is only to suspend the *E. coli*, the *E. coli* cell is not thought to lysed in the solution I step only. Otherwise, RNase might be still stable even in the alkaline condition of solution II, or rapidly renatured to the functional conformations in neutralized condition by solution III. RNase itself is a very stable protein, so we do not have to worry about a loss of enzyme activity at high-temperature. This character of RNase makes us very easy to handle this enzyme in the experiment, but this character often annoys us too, because a contamination of RNase to the other samples completely disturbs our RNA-handling experiment in the laboratory.

Irresponsible usage of RNase often contaminates the laboratory. Therefore, after incubating plasmid sample with RNase, the complete inactivation/removal of RNase should be needed.

5.2. Removal of RNase by phenol/chloroform extraction

Phenol or phenol/chloroform is well known as a protein denaturant. RNase is also inactivated by such as denaturant. Because RNase is very stable, repeating steps of phenol or phenol/chloroform extraction is effective for the complete removal of RNase. On the other hand, this organic reagent often inhibits enzyme activities, once contaminated with the nucleic acids samples. Besides, it is very convincing that phenol or phenol/chloroform have toxicity to cells, resulting in a decrease of transfection efficiency to cultured cells, and so on.

5.3. Salts as an agent for nucleic acid precipitation

It is known that a certain salts selectively precipitate nucleic acids. These salts can be applied to plasmid DNA purification.

5.3.1. Lithium chloride

Lithium chloride (LiCl) at the final concentration of 2.5 M enables us to selectively precipitate RNA. In this condition, RNA makes a pellet by centrifugation, but not DNA. Although low-molecular-weight RNA fragment is not precipitated and remains with plasmid DNA, it is often an adequate quality for using the plasmid in the following experiments, as long as the low-molecular-weight RNA makes critical disturbance for the experiment.

In isolating plasmid DNA by boiling method, Adding LiCl to STET is a better way to do the experiment (see **Figure 2**). After boiling step, centrifugation makes insoluble debris, together with RNA precipitation by the function of LiCl. Therefore, one-step centrifuge is enough for removing protein and RNA. Rather low-molecular-weight RNA still remains in the solution, but normally this RNA does not disturb or inhibit the activity of restriction enzyme and so on.

The insoluble pellet in the boiling method with LiCl is like a chewing gum, and is easily removed by picking with toothpick. Therefore, the combination of boiling method with LiCl is a very reasonable choice. One minor point is that LiCl is rather expensive than Calcium chloride (CaCl_2).

5.3.2. Calcium chloride

Calcium chloride (CaCl_2) is an inexpensive reagent. It is also known to precipitate RNA at the concentration of around 1 M, but DNA is not precipitated in this condition [8]. Thus, this reagent also works in the plasmid purification process like LiCl. However, it is a lack of luckiness that when CaCl_2 is added instead of LiCl in the boiling method, insoluble debris forms crumbly. This means that we cannot pick the debris up by using toothpick, and that we need another tube to transfer the supernatant after centrifugation. This disturbs a merit of the boiling method using only one tube all over the manipulations.

5.4. Polyethylene glycol precipitation of DNA

Polyethylene glycol (PEG) can be used to precipitate DNA [9]. It is also reported that the size of precipitated DNA is controllable by the concentration of PEG [10]. The principle of PEG precipitation is the same as alcohol precipitation, such as ethanol and/or isopropanol. This compound selectively precipitates DNA. Especially, low-molecular-weight RNA, such as transfer RNA, is not precipitated by PEG. There are so many products of PEG, according to their average molecular weights. Generally, PEG #3000, #4000, or #6000 has similar properties for DNA precipitation. Interestingly, to the contrast of that LiCl and CaCl_2 do not precipitate low-molecular-weight RNA, the size between precipitated RNA by salts and non-precipitated RNA by PEG precipitation are complementary in the RNA length from each other.

5.5. Cesium chloride ultracentrifuge

Cesium chloride (CsCl) ultracentrifuge method [11] does not require RNase. It means that phenol/chloroform extraction is not needed in the experiment, so plasmid DNA purified by this method is suitable for the almost all the biochemical experiment. That is, we can apply the plasmid DNA isolated by this method to transfection of the cultured cell and so on.

An ultrapure grade of plasmid is obtained in this method, although a special expensive ultracentrifuge is required for equipment. Moreover, very long time (almost overnight) for centrifuge is needed, and ethidium bromide (EtBr) at a very high concentration (final 800 $\mu\text{g}/\text{mL}$, this is 8000 times higher concentration than agarose gel electrophoresis) is used. EtBr is widely known as a mutagen, and highly concentrated EtBr should be unwanted to handle, if possible. EtBr intercalates to the double-strand of DNA. When this compound is intercalated with DNA, the double-strand of plasmid DNA changes to the slightly unwound form. This affects the sedimentation coefficient of nucleic acids in 10% CsCl solution. Therefore, supercoiled plasmid DNA makes a single sharp band in the tube after ultracentrifugation (200,000 \times g , 20°C, 16 hours). In this experiment, a specially customized tube should be used. The centrifuge tube is made of a soft, translucent plastic polymer, and the plasmid DNA is visualized as

a band in the see-through tube. A syringe needle is inserted into the tube, and the separated plasmid band is sucked into the syringe. After transferring the sucked solution to a new tube, more extra steps are needed to get rid of CsCl and EtBr. This experiment is very sensitive to CsCl concentration. A slight change of CsCl amount causes a negative result; plasmid DNA is not separated as a single band in the tube.

CsCl ultracentrifuge method costs expensive because CsCl is an expensive reagent. Moreover, this experiment is time-consuming, hazardous, and difficult. On the contrary, once succeeded, we can obtain a large amount of ultrapure plasmid DNA. This method can even separate ccDNA from ocDNA, trusting the super high quality of plasmid DNA.

The important fact is that CsCl method is a kind of post-manipulation of alkaline lysis method. That is, alkaline lysis method is such a universal method that it works well as an initial step of CsCl method.

6. Standard plasmid purification method in recent days

Two major kit for plasmid purification is available in the market (**Figure 4**). Both use a basic alkaline lysis method for initial steps, and also uses RNase for RNA removal. A feature of the recent plasmid isolation methods is that they do not go through phenol/chloroform extraction after RNase treatment. Organic solvents are often harmful to cultured cell and so on, so avoiding this reagent in the steps of plasmid purification is a reasonable choice.

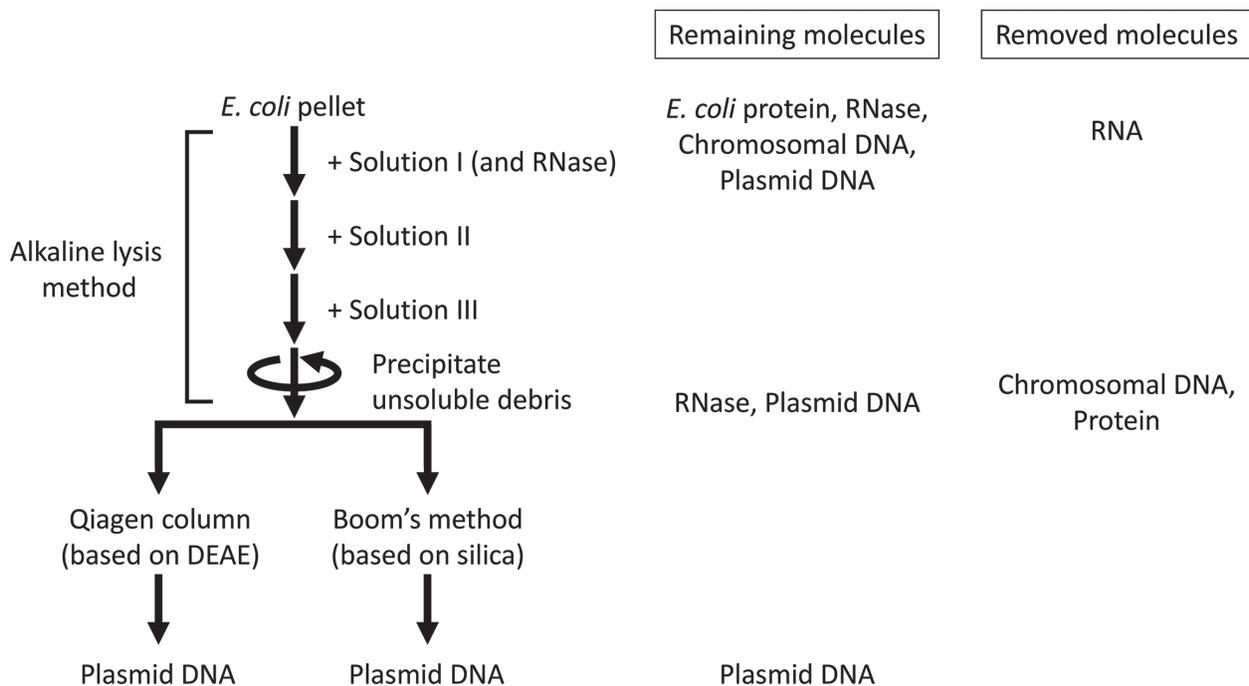


Figure 4. Qiagen kit and silica-membrane kit. They are widely used in many laboratories, but core principle is based on the definitive alkaline lysis method.

6.1. Qiagen column kit

Diethyl-aminoethyl (DEAE) group has positive charges; therefore DEAE-resin is often used to ion-exchange chromatography. DNA also has negative charges, so it binds to DEAE-resin under a certain pH or salt concentration. However, DNA purification by using DEAE was restricted to the recovery step from excised agarose gel and so on, because the binding property of nucleic acids to DEAE is rather broad and weak. Plasmid purification by anion-exchange chromatography has been reported [12], but it will be much better using the column as disposable to avoid contamination of samples. Therefore, DEAE was not seemed to be applicable for plasmid purification in a daily experiment. Qiagen column is famous for its ultrapure quality of purified plasmid DNA, and the principle is ion-exchange column chromatography. The precise information of Qiagen resin is confidential, but basically, it is known that the column consists of a highly condensed anion-exchange group resin [13]. In this experiment, the agent to remove RNA is not an ion-exchange resin, but RNase. A high concentration of RNase is added in the initial step of solution I. During Qiagen chromatography steps, the solutions go through a column in a free fall, so centrifuge step is not required at each step. However, after elution of the plasmid DNA from the resin, the concentration of plasmid DNA may be low, which is inadequate for the following experiment. Therefore, on Qiagen kit, ethanol precipitation is often needed to the eluted solution for concentrating plasmid. It seems that Qiagen columns are suitable for purifying plasmid of mini or maxi scale because the column is rather expensive. Besides, it is not easy to manipulate for multiple open columns at a time.

6.2. Boom's method (silica-membrane kit)

Boom's method is based on a paper and patent by Boom et al. [14], although the principle is widely known as a biochemical property of nucleic acids. The principle of Boom's method is that glass powder or diatomaceous earth (the main ingredient is SiO_2) adsorbs nucleic acids in a chaotropic condition [15], whereas proteins are not. In Boom's method, guanidine hydrochloride or guanidine thiocyanate is often used for chaotropic agent. This adsorption is reversibly eluted by pure water. Hydrophobic condition keeps the adsorption of DNA to SiO_2 , so washing glass powder which adsorbs nucleic acids by 70% ethanol contributes to a high purity of plasmid DNA. The original Boom's method uses grass or diatomaceous earth powder as binding agent, and each step for binding, washing, and eluting is achieved as batch technique (simply centrifuging and discarding the solution). Batch chromatography is very simple method and easy to handle, although pipetting and discarding each solutions makes the experiment rather complicating for manipulating many samples at a time. On the other hand, the commercial kit supplies a column with a silica membrane filter, which is set to 1.5 mL microtube. Once the solution is applied to column, centrifuge step forces the solution go through the column. Therefore, each steps for binding, washing, and eluting needs only several seconds (as much as 1 minute) for centrifugation. Actually, commercial kit of such a silica membrane filter is very easy and useful for handling.

6.2.1. Homemade reagents for Boom's method

The commercial kit supplies their reagents with the column, but the compositions of these reagents are always confidential [16]. On the other hand, the principle of these kits seems

almost the same, based on the DNA adsorption to silica matrix in chaotropic solution. Although it is quite natural to assume that each kit has its own special reagents, homemade solutions based on the original paper are generally available. When applied homemade reagents to commercial silica membrane column, quality and quantity of purified plasmid are almost the same as the commercial kit [17]. It means that these solutions are available by DIY and columns are not still waste, even when reagents in the commercial kit box are expired and out of use.

A modified reagent and modified protocol are also reported to increase the recovery efficiency of the plasmid DNA by commercial column [18]. On the other hand, not silica particles but Zirconium dioxide (ZrO_2 , zirconia) has also been reported as an adsorbent of DNA [19].

6.2.2. Ultra-mini-scale purification

The chaotropic agent is a key factor in Boom's method, so guanidium salt such as GuHCl should play an important role for plasmid DNA purification in Boom's method. But it is reported that guanidium salt is not actually needed for nucleic acids to be adsorbed to silica particles. The other reagent such as high-concentration NaCl also works as chaotropic agent [20]. More surprisingly, a high concentration of the salt in solution III of alkaline lysis method seems already adequate for making the solution to chaotropic condition [21]. It means that adding guanidium for DNA adsorption can be skipped. To cut steps in the experiment has many advantages, especially for handling many samples at a time. Therefore, purifying plasmid samples in 96-well plate without guanidine chaotropic condition is proposed [21], in which method small scale and many samples at a time.

7. 55-minute method

Based on the alkaline lysis method, we developed a new plasmid purification method, which ends within 1 hour and does not need RNase (**Figure 5**) [22]. The principle of this method is a combination of the alkaline lysis method, $CaCl_2$ precipitation, and PEG precipitation. Although a sequential combination of these precipitations was already reported [23], our new invention is that we developed a new composition of solution III. This "super solution III" contains $CaCl_2$ to the standard solution III (Solution III: 5 M $CaCl_2$: H_2O = 2:2:1), which makes not only protein and genomic DNA debris but also a pellet of RNA in the debris. After centrifuging and recovering a supernatant, a standard PEG precipitation makes a plasmid DNA pellet and removes small RNA, which was not precipitated at the super solution III step. After all, only plasmid DNA remains in the final solution. Actually, this method needs totally 55 minutes from collecting *E. coli* pellet to recovering the final purified plasmid DNA. The great advantage of this method is that we are able to eliminate the use of RNase. Therefore, even RNase removal step is also eliminated. A quality and quantity are adequate for doing another experiment such as transfection into cultured cells.

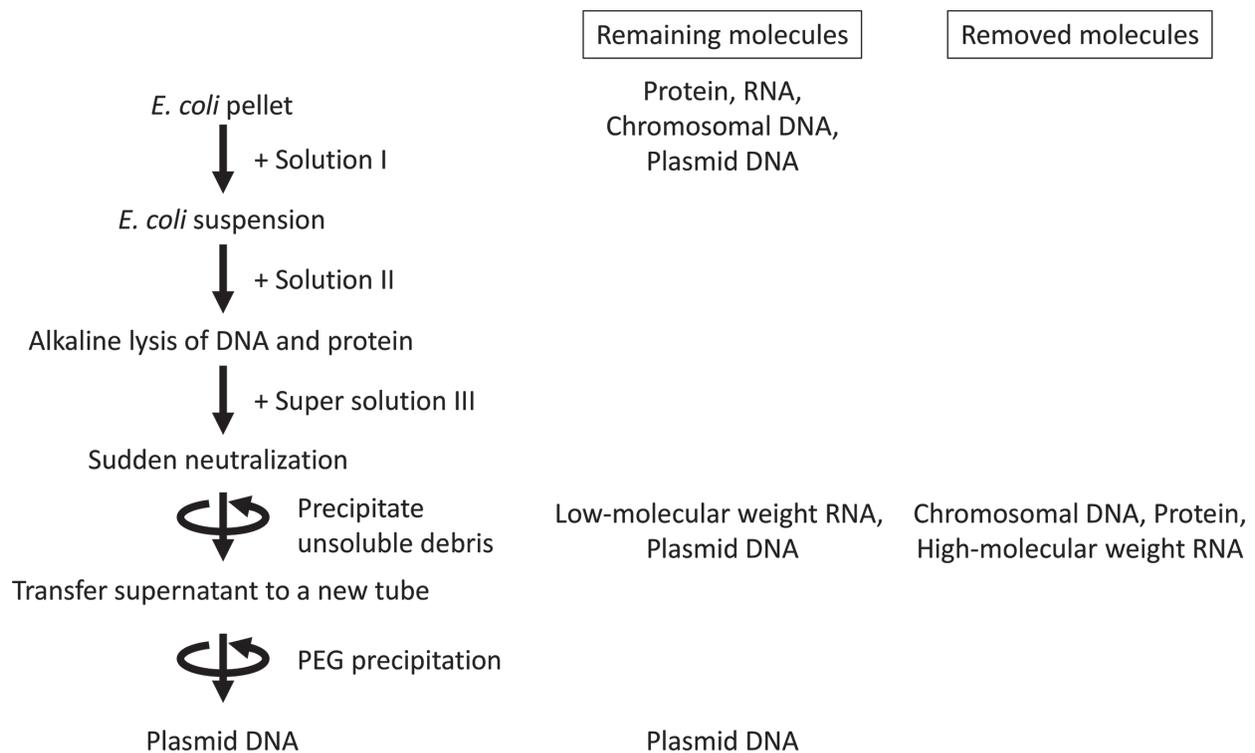


Figure 5. Scheme for 55 minutes method [17]. Note that it does not need to use RNase for RNA removal.

8. Conclusion

In a course of doing plasmid purification for every day, I noticed several tips for the experiment.

- i. Alkaline denaturation/renaturation steps are so sophisticated that it is the definitive method for plasmid purification. None of another method will take over the alkaline lysis method. However, RNA is not removed in alkaline lysis method, so RNA removal steps should be applied in a course of plasmid isolation by alkaline lysis method.
- ii. RNase is an easy choice to remove RNA, but should be completely removed after RNA digestion. One of the solutions is phenol/chloroform protein extraction, but phenol/chloroform may play a troublesome factor. Only a slight contamination of this reagent inhibits the activities of several enzymes and disturbs biochemical experiments. It also has toxicity to the cells, also disturbing transfection experiments. In other words, eliminating phenol/chloroform step in plasmid purification is the key point to trust its purity.
- iii. Qiagen kits and Silica-membrane kits are actually the extra steps after alkaline lysis method. These kits need RNase for RNA digest. In other words, they work as RNase remover from the solution.
- iv. RNase completely digests unwanted RNA from the plasmid sample. But this enzyme is very stable and very hard to inactivate, even disturbing RNA experiment in the laboratory.

Moreover, RNase is usually isolated from animals such as bovine, which may induce allergy to the human in gene therapy [24].

Based on these tips, we developed a new composition of solution III on alkaline lysis method, which enables us to purify plasmid DNA without adding of RNase. This method does not need any special columns or resins, but plasmid DNA purified by this method has enough quality for applying transfection to the cultured cell, injection into the nematode, and so on. Our result indicates that plasmid DNA purification without phenol/chloroform extraction is a great advantage for the quality of purified plasmid DNA.

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Conflict of interest

The author has no conflicts of interest directly relevant to the content of this article.

Notes/Thanks/Other declarations

The author has no other declarations about this article.

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