Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6988

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (5):** 533-541 (2018)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

A Comparative Study on Different Plasmid Isolation Procedures

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Received: 18.07.2018 | Revised: 24.08.2018 | Accepted: 30.08.2018

ABSTRACT

Plasmid DNA has several applications in the field of modern biotechnology. Despite the presence of several methods to remove contaminants and/ or to purify plasmid DNA from primary isolate, the first technique of plasmid isolation determines the quality of the final product. In this study, some popular methods of plasmid DNA isolation have been compared in terms of their rapidity, plasmid quality and ease of experiments. Three different STET buffers used in boiling lysis process were also compared to identify the best performing one. Highest quantity of RNA contaminants, as well as plasmid, was isolated in the alkaline extraction process. Best supercoiled plasmid percentage was isolated by boiling lysis STET buffer 2. The RE digestion was non-reactive/ improper in boiling lysis methods whereas it was perfect in alkaline lysis despite huge quantity of RNA contaminants. Alkaline lysis was proved to be the best one among all and with some further purification processes indicated, other processes can also be used efficiently.

Key words: *Alkaline lysis, Boiling lysis, STET buffer, Plasmid isolation, Plasmid quality*

Abbreviations-

BL1: Plasmid isolation by boiling lysis process with STET 1 buffer
BL2: Plasmid isolation by boiling lysis process with STET 2 buffer
BL3: Plasmid isolation by boiling lysis process with STET 3 buffer

INTRODUCTION

Plasmid is extra chromosomal DNA that exists mainly in prokaryotes like bacteria and archaea. However, extra chromosomal plasmids also exist in certain eukaryotic fungal species[30]. These plasmids exhibit many interesting characteristics like antimicrobial resistance or fertility factor in the life of the host organism. Replication of plasmid does not depend upon the host chromosomal DNA machinery but rather follows different approaches[26]. Being a small (2-20 kb in average) independent DNA molecule, plasmid DNA has been the centre of the biotechnological studies for a long time. Plasmid DNAs are easily modifiable using genetic engineering approach and these can be maintained and replicated in a host bacterial organism (like *Escherichia coli*).

Cite this article: Roy, U., Mishra, A., Jana, P. and Karmakar, S., A Comparative Study on Different Plasmid Isolation Procedures, *Int. J. Pure App. Biosci.* **6(5):** 533-541 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6988

In modern days, plasmid DNAs are used in thousands of ways including sequencing, gene expression, promoter analysis, gene therapy, DNA vaccine, gene knockdown/ knockout, CRISPR-Cas, production of transgenic organism and many more. Successes of these applications depend upon the quality of the plasmid DNA. Plasmids exist in a complex topologically intertwined supercoiled structure inside the cell. Due to the mechanical or shear stress applied during extraction, the plasmid comes in some other forms like open circular, linear, covalently bounded (figure 1). But, the supercoiled plasmids are mostly accepted by cells during transformation or transfection processes. The final plasmid should be free from any traces of contaminants like degraded RNA, genomic DNA, bacterial proteins and endotoxins. There are several approaches to remove these contaminants like use of chloride salts to remove high molecular weight RNA[7,29], selective precipitation of supercoiled plasmid[21,25,29,32],filtration[12] and chromatographic techniques[11]. Though all these techniques are standardized over time to work efficiently, the primary technique of plasmid isolation determines the quality of the final product.

In this study, we have compared some popular methods of plasmid extraction in terms of their rapidity, plasmid quality and ease of experiments. Alkaline extraction is the most popular method of plasmid extraction till date. In this process, the bacterial cell is ruptured by exposing it to a highly alkaline solution along with detergent. The gDNA and cellular proteins are selectively precipitated by addition of an acidic neutralization solution while the topologically intertwined plasmids are retained in the aqueous phase[2,26]. Boiling lysis is another popular method of plasmid isolation in which cells are ruptured with a combination of detergent, lysozyme and a heat shock. The chromosomal DNA remains attached to bacterial proteins and is precipitated by centrifugation while the plasmid remains in the supernatant[18]. The lysis buffer used in boiling lysis method somewhat differs in available different literature. In this study, we have also compared all the different compositions to find out the best working one.

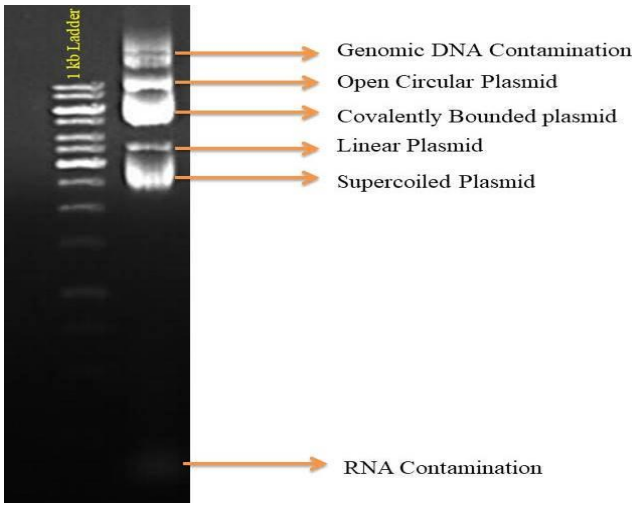

Figure 1: Different contaminants and isoforms of plasmids after plasmid extraction process. Photograph obtained after 1% agarose gel electrophoresis with voltage of 5 volt/cm of gel for 1.5 hours.

MATERIAL AND METHODS

Bacterial culture

E.coli (strain DH5α), harbouring a kanamycin resistance gene containing plasmid was cultured in Luria bertani broth with 50µg/ ml of kanamycin selection pressure at 37°C in a shaking incubator at 160 rpm overnight (16 hours). The final OD_{600} of the culture was 1.12. The same wet weight of bacterial pellet (20 mg) was taken in all the experiments.

Plasmid isolation

The plasmid was extracted mainly by two methods. The alkaline extraction procedure was carried out according to Sambrook *et al*.[26]. The boiling lysis method was carried out using three different lysis buffers (named STET buffer 1, 2 & 3). The composition of the buffer system is given in table no. 1. As a positive control, plasmid was also isolated using Qiagen miniprep kit following the manufacturer's protocol. All the experiments were carried out in triplicates.

Table 1: Comparison of different STET buffer compositions

Buffer	Composition	Reference
STET 1	8% Sucrose, 5% Triton X-100, 50 mM EDTA (pH-8), 50 mM Tris-Cl (pH-8). The solution is autoclaved and stored at 4°C.	18
STET 2	8% Sucrose, 0.5% Triton X-100, 50mM EDTA (pH-8), 10mM Tris-Cl (pH-8). The solution is autoclaved and stored at 4°C.	This study
STET 3	10mM Tris-Cl (pH-8), 0.1M NaCl, 1mM EDTA (pH-8), 5% v/v Triton X-100. pH of STET is 8.0 after all the ingredients are added. No need of sterilize before use.	26

Procedure for plasmid isolation in boiling lysis was carried out according to Sambrook *et al*.[26] with some minor modifications. Briefly, 20 mg (wet weight) of bacterial pellet was washed with PBS and resuspended in 350µl of STET buffer by slow vortexing. Resuspended solutions were incubated in ice for 5 minutes. 25µl of freshly prepared lysozyme solution (10 mg/ ml in 10mM Tris-cl, pH 8.0) was added to the resuspended bacterial solution. After thorough mixing, the tubes were kept in a boiling water bath (100°C) for exactly 40 seconds and immediately kept on ice for 2 minutes. The tubes were then centrifuged at 14000 rpm for 20 minutes at 4°C. The resultant supernatant was decanted in another tube, rather than pipetting out because pipetting can sometimes result in entry of viscous protein containing liquid. But, decanting is only recommended after application of centrifugation time and speed mentioned above. 0.6 volume of room temperature isopropanol was added to the supernatant, incubated at room temperature for 15 minutes and centrifuged at 12000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed twice with cold 70% alcohol. Centrifugation after alcohol wash was avoided. Lastly, the pellets were resuspended in 30µl of TE buffer.

Plasmid quality determination

The plasmid quality was determined depending upon their plasmid yield, 260/280 ratio, RNA contamination, genomic DNA contamination, supercoiled plasmid percentage and RE digestion. Plasmid yield and 260/280 ratio was determined by spectrophotometric method in nan drop. RNA contamination was estimated by 1% agarose gel electrophoresis. Genomic DNA contamination percentage and supercoiled plasmid DNA percentage was determined using my Image Analysis software (Thermo Scientific, USA) by analysing the gel image. Double RE digestion [using Xho1 and Sph1 (Thermo Scientific, USA)] was performed to identify the presence of enzyme inhibitory contaminants in the isolate. RE digestion was performed at 37°C for 3 hours without RNase treatment.

RESULTS

Plasmid yield

The boiling lysis procedure took almost 1 hour 30 minutes whereas the alkaline lysis method took nearly 2 hours and the Qiagen miniprep kit took 30 minutes to be completed. In BL1, BL2, BL3, alkaline lysis and Qiagen miniprep kit, the total plasmids extracted are 33.6µg, 62.21µg, 57.7µg, 86µg and 7.6µg respectively. Among the traditional methods, the best 260/280 ratio (near to 1.8) was obtained in BL1 method. The supercoiled percentage was highest in BL2 method (34.32%). Alkaline lysis and BL3 methods showed the highest quantity of genomic DNA contamination (shown in figure 2). The other quality data are noted in table 2.

Table 2: Quality comparison of plasmid isolated by different processes

Processes / Quality	Boiling Lysis STET 1	Boiling Lysis STET 2	Boiling Lysis STET 3	Alkaline Lysis	Qiagen Miniprep
Plasmid yield (µg)	33.6	62.1	57.7	86	7.6
260/280	1.935	2.075	2.04	2.168	1.801
Supercoiled %	33.4	34.32	32.66	27	30
Genomic DNA contamination	Low	Low	High	High	Low
RE digestion	Negative	Negative	Partial	Positive	Positive

RE digestion

The RE digestion was performed using Xho1 and Sph1 enzymes at 37°C for 3 hours. The expected fragment sizes were 1.2 kb and 2.0 kb. No bands were seen in case of BL1 and BL2 plasmids after RE digestion. In the case of BL 3 plasmid, an improper RE was observed which provided faint bands of expected sizes. The plasmids isolated by alkaline lysis and Qiagen miniprep kit were digested perfectly (figure 3).

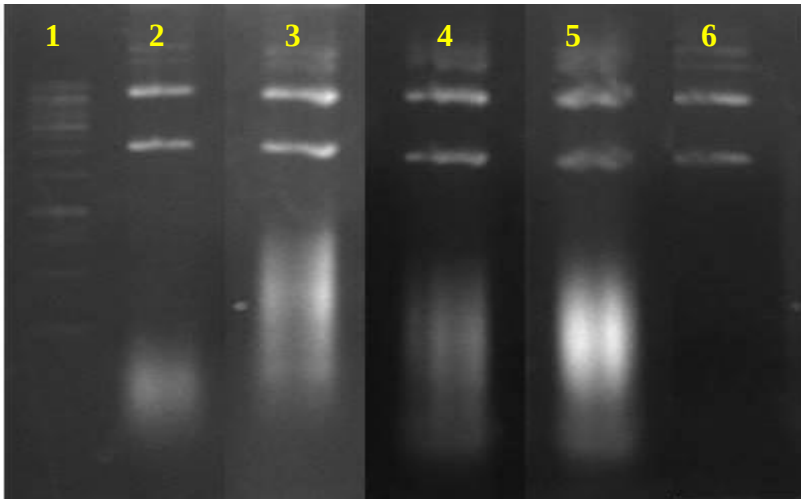

Figure 2: Agarose gel electrophoresis image of plasmids isolated by different procedures. 1 kb DNA ladder (lane 1); plasmid isolated by BL1 method (lane 2); plasmid isolated by BL2 method (lane 3); plasmid isolated by BL3 method (lane 4); plasmid isolated by alkaline lysis procedure (lane 5); plasmid isolated by Qiagen miniprep kit (lane 6).

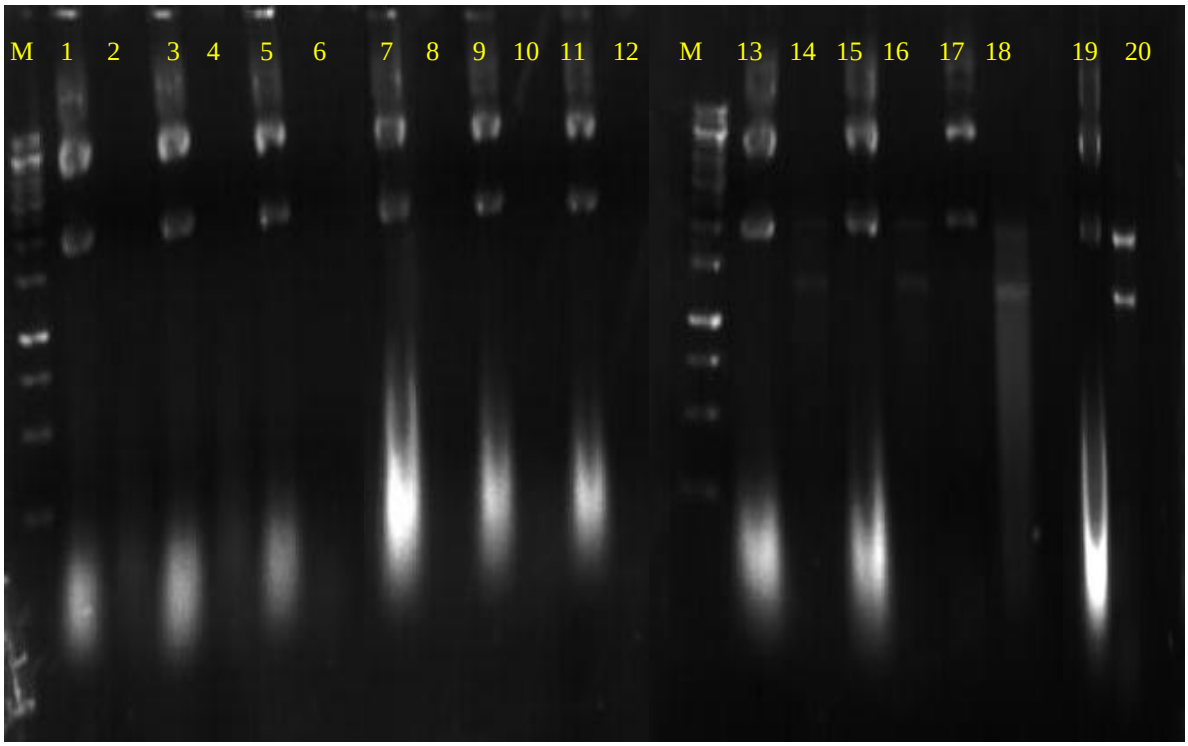

Figure 3: RE digestion of plasmids.
1 kb DNA ladder (lane M); plasmids isolated by BL1 (lane 1, 3, 5); RE of plasmids isolated by BL1 (lane 2, 4,6); plasmids isolated by BL2 (lane 7, 9, 11); RE of plasmids isolated by BL2 (lane 8, 10, 12); plasmids isolated by BL3 (lane 13, 15, 17); RE of plasmids isolated by BL3 (lane 14, 16, 18); plasmid isolated by alkaline lysis (lane 19); RE of plasmid extracted by alkaline lysis (lane 20).

DISCUSSIONS

In the current study, we compared some popular methods for plasmid isolation to find out the most efficient one in terms of rapidity, plasmid quality and ease of experiment. Although the Qiagen miniprep kit follows the basic principle of alkaline lysis method, it is far more rapid than the traditional one because it contains RNase in the resuspension buffer solution and it omits most of the incubation steps to be followed in the traditional process. The boiling lysis method is sufficiently faster (1.5 hours) than the alkaline lysis method (2 hours). However, the former one needs instruments like boiling water bath which may not be a common instrument in common molecular biology laboratory. Furthermore, the time required for alkaline lysis process can be reduced by minimizing or omitting some of the incubation steps.

The spectrophotometric highest yield of plasmid was obtained in alkaline lysis method which was confirmed by agarose gel electrophoresis image (figure 2). Subsequently, the lowest yield was in Qiagen miniprep kit as the capacity of the silica column used in the kit is limited to 10 µg only. BL 1 yielded somewhat lower yield than the other two methods in spectrophotometric assay, but the result was not in line with the gel image (figure 2). The purity (260/280 ratio) of the isolated plasmid can solve this biases as the purity of the BL1 plasmid was somewhat higher than the others (table 2). It is possible that the contaminants in BL2 and BL3 plasmids probably contributed in misreading the actual absorption. The alkaline lysis isolated plasmids were the most RNA contaminated ones which can be seen by its high 260/280 ratio (2.168) and the large smear in gel image (figure 2). The boiling lysis method is not recommended when the copy number of plasmid is low as its efficiency is lower than alkaline lysis[9,26]. Our result also

indicated lower yield in boiling lysis procedures. The yield was sufficiently high because the plasmid used by us has high copy number pUC origin of replication.

The supercoiled plasmid percentage was higher in all the boiling lysis methods with BL2 being the highest (34.32%). In contrast, the alkaline lysis and the kit method both possessed a lower percentage of supercoiled plasmid (table 2). The cell lysis period in alkaline lysis period and mixing pattern after addition of lysis buffer are very crucial steps for good quality supercoiled plasmid isolation[26]. The standard incubation period for lysis in alkaline lysis is 5 minutes. However, the lysis time can vary depending upon the strain of bacteria, amount of bacteria and mixing. Careful standardization of lysis time according to the mentioned factors can help to yield more supercoiled DNA.

BL1 and BL2 plasmids failed to be digested properly in restriction analysis whereas the BL3 plasmids digested partially. The bacterial species that release carbohydrates in presence of detergent, lysozyme and heat (HB101) and the endonuclease A (*endA*+) strains (HB101, JM100) are not suitable for plasmid extraction by boiling lysis procedure[9,26]. Contamination by endonuclease is not completely removed during boiling and can result in complete plasmid degradation when RE digestion is performed in presence of Mg2+ ion[9]. Although DH5α is neither a carbohydrate releasing or *endA*+ strain, it is suspected that the presence of other endonucleases has caused the complete disappearance of the plasmid. The BL3 method contained somewhat less quantity of endonuclease and showed improper RE digestion. In contrast, the plasmid isolated by alkaline lysis method was properly digested by restriction enzymes, indicating that although the isolate had a huge quantity of RNA contamination; it was completely free from endonucleases or any other enzyme inhibitory substances.

The problem of contamination can be avoided by certain additional steps in all the traditional processes. An additional phenol-chloroform extraction step can be implemented after the first supernatant isolation in boiling lysis procedure to get rid of endonuclease problem[9]. The contamination of RNA in alkaline extraction can be completely removed by a brief treatment with RNase A. But, both the phenol and RNase A can be unsuitable for in vivo applications like gene therapy or DNA vaccination[4,13] because the phenol is a corrosive agent and R Nase is of bovine origin and can carry unnecessary harmful viruses.

There are several methods for selective purification of supercoiled plasmid DNA after isolation. Density gradient centrifugation using cesium chloride and ethidium bromide was one of the first methods of purifying supercoiled plasmid DNA[14]. However, the yield is low in this technique and both cesium chloride and ethidium bromide are toxic for cells. Because of these restrictions, density gradient centrifugation is rarely used for plasmid purification now. Removal of RNA contamination from plasmid DNA solution has been successfully carried out by selective precipitation with calcium chloride[29], lithium, ammonium acetate[19] etc. or by filtration techniques like tangential flow filtration[12,16,24,31]. Selective precipitation of supercoiled plasmid DNA can be achieved by use of polyethylene glycol (PEG)[29], cetyltrimethylammonium bromide (CTAB)[21], polyamines[25], poly (*N*, *N*-dimethyldiallylammonium) chloride[32] etc. Chromatographic techniques are the modern ones used to selectively purify supercoiled plasmid DNA. Although many types of chromatography including size exclusion[6,22], hydrophobic interaction[8], hydroxyapatite[15], thiophilic interaction[27], anion exchange[11] are used, anion exchange chromatography proved to be the most successful and efficient one. Techniques like monolith columns[1,5,17,20] and chromatographic capsules[10] are being increasingly popular over traditional chromatographic columns. Mostly, combinations of different techniques are applied for ease of purification. For small scale applications, homemade filtration column[28] or

homemade silica columns[3,23] can be efficiently used.

From this study, we conclude that tough the alkaline lysis process produces a lot of RNA contamination, the plasmid yield is way higher than other procedures tested. Moreover, the alkaline lysis plasmid is completely free from any enzyme inhibitory compounds. The boiling lysis procedure can be unsuitable for enzymatic reactions without any additional treatment. Any purification technique or combination of purification techniques mentioned above can be used effectively to further purify the DNA for use in other applications.

Acknowledgement

The authors would like to acknowledge the Director, Indian Council of Agricultural Research-Central Institute of Fisheries Education, Mumbai for providing the necessary facilities.

Conflict of interest

The authors declare that they do not have any conflict of interest.

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