

A high-salinity solution with calcium chloride enables RNase-free, easy plasmid isolation within 55 minutes

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Summary

We dramatically improved a plasmid-isolation protocol based on the popular alkaline-sodium dodecyl sulfate plasmid isolation method. Our modified method provides significant time and cost savings. We used a modified solution during the neutralization step, which allowed us to skip several subsequent handling steps, saving a great amount of time. The plasmids purified by this method were of high quality, and the optical density ratio 260 and 280 was approximately 1.8. Plasmid DNA isolated by our method was of sufficient quality to perform subsequent restriction enzyme cuts and other downstream experiments, including budding yeast transformation, cultured cell transfection, and *Caenorhabditis elegans* injection experiments.

Keywords: Plasmid isolation, calcium chloride, polyethylene glycol, RNase-free

1. Introduction

Plasmid isolation from *Escherichia coli* is an indispensable step in most routine laboratory experiments for molecular biology, biochemistry, and cell biology. There are several published plasmid-isolation methods (1-8). Among them, the alkaline-sodium dodecyl sulfate (SDS) method (1) is the most popular procedure for purifying plasmid DNA. In this method, the DNA denaturation step (using Solution II) and neutralization step (using Solution III) are very effective and sophisticated techniques for separating plasmid DNA from *E. coli* genomic DNA. Moreover, insoluble cellular debris, including proteins, is separated together with genomic DNA from plasmids. One of the difficulties of this popular method is that a huge amount of RNA is collected along with the plasmid DNA. Therefore, RNase is always required to remove unwanted RNA from the plasmid solution. Then a hazardous organic solvent (phenol/chloroform) is added to inactivate and remove the RNase protein. This process requires several additional steps and extra time.

Many commercial kits are available for plasmid isolation. Two major kits with different principles are widely used. One is based on an anion-exchange resin (9). Plasmid DNA is adsorbed onto the resin by the negative charge of DNA and then eluted by adding a high-salinity solution. Another method employs a silica membrane with chaotropic solutions (10,11). Under chaotropic conditions, nucleic acids are adsorbed onto silica particles and eluted using pure water. In both major commercial kits, the principle of separating plasmid DNA from bacterial genomic DNA is still based on the popular alkaline-SDS method. Moreover, both kits require RNase to digest unwanted RNA. Therefore, a large amount of RNase is added to the kit solution. These kits are very easy to use, but rather expensive. Thus, another time- and cost-saving protocol for high-quality and high-quantity plasmid isolation is needed for everyday experiments in the laboratory. Furthermore, RNase is widely known as a robust, stable protein. RNase protein contamination results in the degradation of RNA in the laboratory and disrupts RNA experiments. It is best not to use RNase protein in laboratories that handle RNA molecules.

Calcium chloride (CaCl₂) is an effective reagent that selectively removes RNA from a mixture of DNA and RNA (12,13). That is, RNA can be precipitated by centrifugation in the presence of CaCl₂ (RNase is not needed). However, this requires several centrifugation

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steps and takes quite a long time. Hence, we established a modified plasmid purification method using CaCl_2 (called the Super Sol III method or Sasagawa method) that is based on standard alkaline-SDS isolation but is much easier and less time consuming. Our method eliminates several steps, allowing us to isolate plasmid DNA in much less time, and the total isolation time is around 55 min.

2. Materials and Methods

2.1. *E. coli*, liquid medium, and plasmid DNA

We used *E. coli* JM109 or XL-1 blue, which we routinely use for cloning experiments. Bacteria were grown in LB medium supplemented with ampicillin (final concentration, 50 $\mu\text{L}/\text{mL}$). *E. coli* was incubated in 15-50-mL tubes with 5-10 mL LB medium in a shaking air incubator. The plasmids pBluescript and pUC118, and their derivatives, were also tested.

2.2. Reagents and equipment

For all of our experiments, the purest grade reagents available were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). Solutions I and II were prepared according to a standard protocol (14). We purchased restriction enzymes from Takara-Bio (Shiga, Japan) and/or Toyobo (Osaka, Japan) to cut plasmid DNA. Sample quality and quantity were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Yokohama, Japan). A Narishige micromanipulator (Tokyo, Japan) was used to inject plasmid into *C. elegans*.

2.3. Super Sol III solution

The modified Solution III (Super sol III or Sol III-Ca) consisted of 1 mL Solution III (14), 1 mL 5 M CaCl_2 , and 0.5 mL H_2O . A warming step to dissolve the solution might be needed (*i.e.*, 37°C or above).

2.4. Handmade filtration column

We made a filtration column as follows (Figure 1). The bottom of a 5-mL polystyrene round-bottom tube (*e.g.*, Falcon 352058) was drilled using a heated ice pick to create a pinhole (Figure 1a). A polypropylene centrifuge tube (15 mL) was used for sample collection. We made a hole in a 15-mL screw cap tube using a cork borer (#6, $\Phi 12$ mm). The round-bottom tube was inserted through the cap hole, and the top of the round-bottom tube was taped with electrical tape as a stopper (Figure 1b). A piece of tissue paper was pushed firmly into a 5-mL polystyrene round-bottom tube (Figure 1c). Before use, 5 mL H_2O was applied and the filter was washed using centrifugation. After

centrifugation, filtered H_2O was discarded.

2.5. Budding yeast, cultured cells, and *C. elegans*

The budding yeast *Saccharomyces cerevisiae* strain PJ69-4A was used for transformation. A derivative of plasmid p426ADH (15) was transformed into the yeast using URA3 as a selectable marker. A pEGFP plasmid was transfected into HeLa cells as a model of mammalian cell transfection. *C. elegans* (N2 strain) were injected with plasmids pRF4, in which a mutated collagen gene is coded.

3. Results

3.1. Removal of high-molecular-weight RNA in a single step

Our first challenge was to modify Solution III by adding CaCl_2 . We named the new mixture Super Sol III. We tested it and found that it neutralized as well as traditional Solution III. Moreover, it greatly reduced the amount of RNA in the cleared lysate. Higher molecular weight RNA precipitates out with protein and genomic DNA. Only small RNAs, such as tRNA, seem to remain in the lysate (Figure 2, lane 3).

Centrifugation was thought to be necessary to precipitate RNA in the presence of CaCl_2 . However, surprisingly, we found that centrifugation was not absolutely necessary to remove high-molecular-weight RNA. To test the effect of Super Sol III on RNA removal from the lysate, we first filtered the neutralized sample solution, which contained a large amount of debris, without using centrifugation. We then added 2-propanol to the filtered lysate and centrifuged the

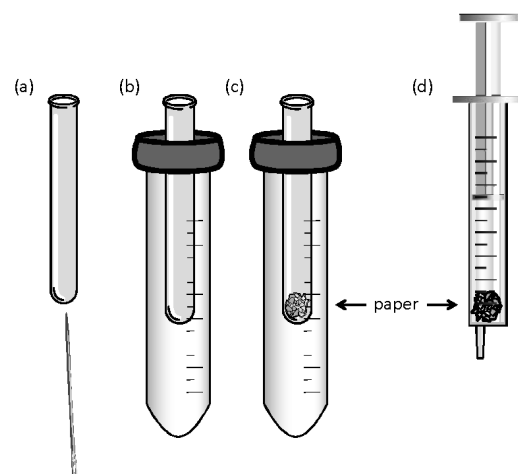


Figure 1. A handmade filtration column. (a) A hole was made in the bottom of a round-bottom 5-mL polystyrene tube using a heated needle. (b) A hole was made in the top of a 15-mL tube screw cap, and a 5-mL tube (a) was inserted through the hole. The top of the round-bottom tube was taped with electrical tape as a stopper. (c) A piece of tissue paper was pushed into a 5-mL polystyrene round-bottom tube. (d) A 2.5-10-mL disposable syringe with tissue paper could also be used as a handmade filter in this experiment.

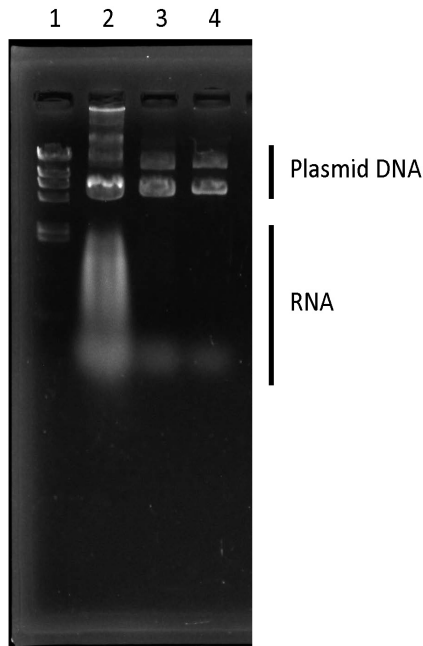


Figure 2. Effect of RNA removal by CaCl_2 , which is premixed into Solution III. Lane 1: molecular weight marker. Lane 2: negative control (standard alkali-SDS plasmid purification without RNase). Lane 3: a solution neutralized by Super Sol III was centrifuged, and then 2-propanol precipitation was performed. Lane 4: a solution neutralized by Super Sol III was filtered without centrifugation, and then 2-propanol precipitation was performed.

sample to precipitate nucleic acids. This procedure resulted in the recovery of plasmid DNA and a small amount of RNA (Figure 2, lane 4). These results indicate that a large amount of RNA became insoluble, and was removed with insoluble proteins and debris as well as genomic DNA during the filtration step.

3.2. A combination of Super Sol III and polyethylene glycol precipitation

Based on the above results, we further improved the protocol by adding polyethylene glycol directly to the filtered lysate to precipitate plasmid DNA. Polyethylene glycol precipitates plasmid DNA, but not small-molecular-weight RNA (16,17). We added polyethylene glycol to a final concentration of 0-12% to filtered lysate and centrifuged the sample to precipitate plasmid DNA. Pure plasmid DNA without unwanted RNA was obtained. Even small-molecular-weight RNA disappeared from the sample (Figure 3). A final concentration of 6-12% polyethylene glycol produced good results. Therefore, we decided to precipitate plasmid DNA to a final concentration of 8% polyethylene glycol by adding 32% polyethylene glycol solution. Both polyethylene glycol #4,000 and #6,000 worked well for precipitating clear plasmid DNA (data not shown).

Prior to polyethylene glycol precipitation, the debris (*i.e.*, insoluble proteins and genomic DNA) should be completely removed from the lysate. For this purpose,

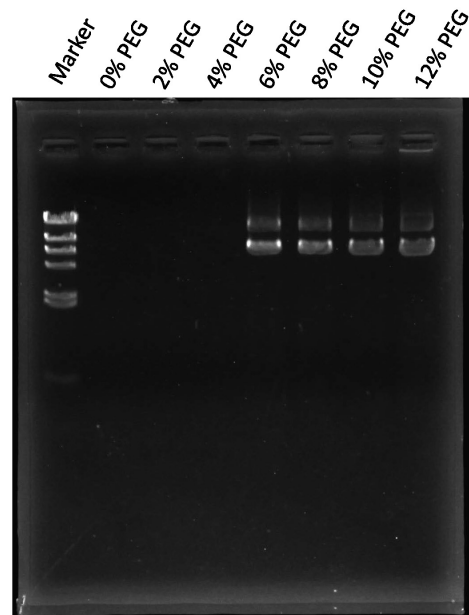


Figure 3. A combination of Super Sol III and polyethylene glycol precipitation. Lane 1: molecular weight marker. Lanes 2–8 represent various final concentrations of polyethylene glycol (PEG) during the plasmid precipitation step. Lane 2: 0% PEG. Lane 3: 2% PEG. Lane 4: 4% PEG. Lane 5: 6% PEG. Lane 6: 8% PEG. Lane 7: 10% PEG. Lane 8: 12% PEG.

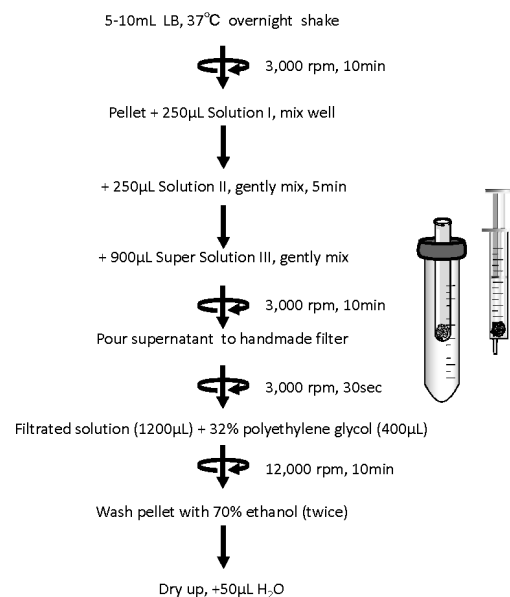


Figure 4. The complete 55-min protocol established in this study.

we made a handmade filtration column (Figure 1). Based on these results, we established our complete plasmid purification protocol (Figure 4).

3.3. Quality and quantity check of plasmid DNA by spectrophotometer

We checked the quality of plasmid DNA purified by our Super Sol III method. As shown in Table 1, we obtained very high quality plasmid DNA. The optical

Table 1. Spectrophotometric DNA quality and quantity check

	A ₂₆₀	Conc.(ng/μL)	Total plasmid (μg)	A ₂₆₀ /A ₂₈₀
Average	3.58	179	8.95	1.85
S.D	1.81	91	4.54	0.04

Data are presented as average ± S.D.

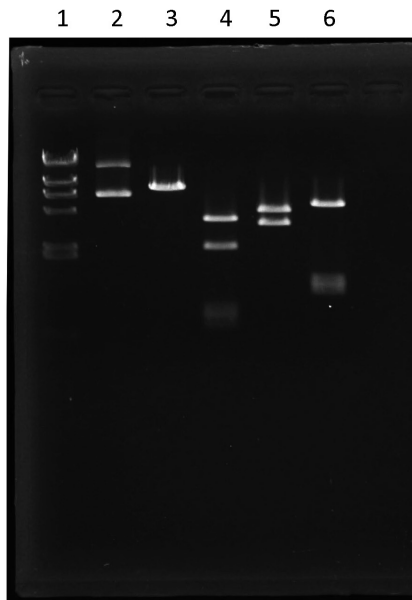


Figure 5. Restriction enzyme check. Lane 1: molecular weight marker. Lane 2: uncut plasmid DNA. Lane 3: *Kpn* I cut (Low-salt buffer). Lane 4: *Hind* III cut (Medium-salt buffer). Lane 5: *Eco* R I cut (High-salt buffer). Lane 6: *Bam* H I cut (High-salt buffer with potassium).

density ratio at 260 and 280 (A₂₆₀/A₂₈₀) was around 1.8, indicating that there was no protein contamination in the isolated plasmid. The quantity of plasmid DNA was almost 1 μg/mL in LB medium, which is sufficient for downstream experiments. We found that both handmade-columns and syringe filters have good qualities (Table 1).

3.4. Restriction enzyme check

The quality of purified plasmid DNA was also checked by using restriction enzymes. A purified plasmid was cut with *Eco* R I (High-salt buffer), *Bam* H I (High-salt buffer with potassium), *Hind* III (Medium-salt buffer), and *Kpn* I (Low-salt buffer). All of these enzymes successfully cut plasmid DNA (Figure 5).

3.5. Injection into *C. elegans*

To check the quality of the purified plasmid, we injected plasmids into *C. elegans* following a previous study (18). Generally, we obtain F1 transformants in the injection experiments, but the F2 transformant (*i.e.* stable transformant) appears only when the transgene is provided in the F1 germline. That is, we can conclude the stable transformation experiment was successful

Table 2. Results of DNA injection into *C. elegans*

Injected worm	9
F1 transformant	6
F2 transformant	3

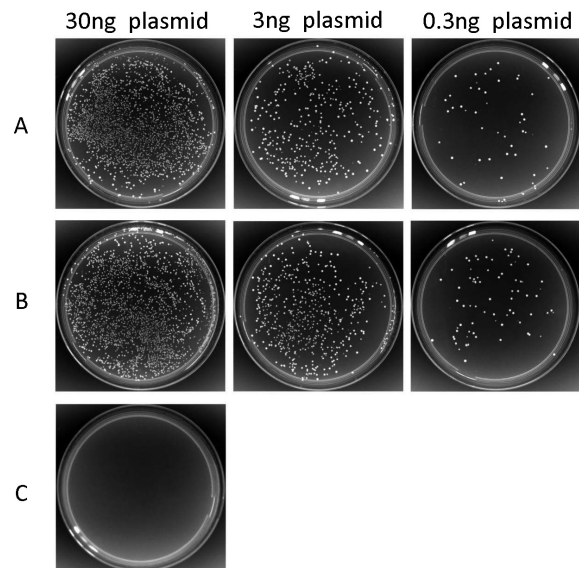


Figure 6. Results of yeast transformation. In total 30 ng, 3 ng, and 0.3 ng plasmid per plate were transformed into the budding yeast. (A), plasmid DNA isolated by our protocol. (B), plasmid DNA isolated by a standard silica membrane kit (positive control). (C), no plasmid DNA (negative control).

only when we obtained F2 transformants. We injected plasmid DNA from our method into nine worms, and obtained six F1 transformants. And then, one of the F1 transformants had F2 transformants, indicating that our injection experiments were successful using the plasmid purified by our method (Table 2).

3.6. Yeast transformation

We performed yeast transformation in *S. cerevisiae* to determine plasmid DNA quality. A derivative of plasmid p426ADH (15) was purified using our plasmid isolation method, and then transformed into *S. cerevisiae* with a standard lithium chloride protocol (19). As shown in Figure 6, transformation was efficient with our plasmid, and was similar to the plasmid transformation efficiency achieved when using a Sigma commercial kit (10). Transformation efficiency was calculated as colony forming units per 1 μg plasmid (cfu/μg). According to this calculation, the efficiency of our system was 1.8×10^5 cfu/μg, while the efficiency using the Sigma kit was 2.5×10^5 cfu/μg. Generally, around 10^5 cfu/μg is an acceptable result.

3.7. Transfection into cultured cells

Our plasmid-isolation method also provided plasmids of sufficient quality for transfection into cultured cells.

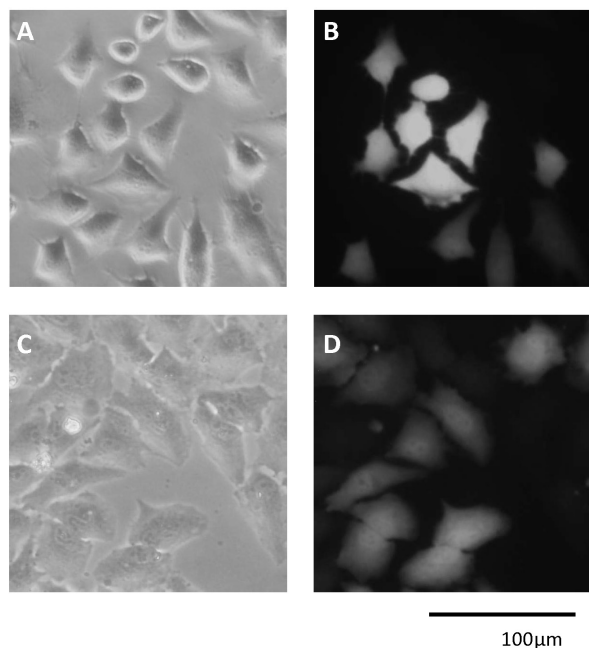


Figure 7. Result of cultured cell transfection. Upper panels (A, B) are cells transfected using a plasmid described in this manuscript, and lower panels (C, D) are cells using a commercial kit. (A) and (C), phase-contrast image; (B) and (D), EGFP fluorescence detection.

Using the standard Fugene 6.0 (Promega) protocol (20), 0.5 μg pEGFP plasmid was transfected into cells seeded in a 20-mm dish. As shown in Figure 7, we successfully observed EGFP fluorescence in HeLa cells. This indicates that plasmid DNA isolated by our method is of sufficient quality for use in cell transfection. We counted cells in microscopic images and calculated transfection efficiencies. The transfection efficiency of our plasmid isolation method was 20%, whereas the efficiency was 50% when we transfected a plasmid with a Sigma kit.

4. Discussion

The advantage of the traditional alkaline-SDS method is that chromosomal *E. coli* DNA is removed, along with insoluble debris, by several simple steps, leaving plasmid DNA in the cleared lysate. The basic steps of this traditional method include cell lysis and protein/DNA denaturation by the alkaline solution (Sol II) and a sudden pH change to neutrality by neutralization buffer (Sol III). This sudden pH change is essential to transform genomic DNA and proteins into insoluble debris.

The difficulty of this method lies in separating RNA from the cleared lysate; RNA and plasmid DNA react similarly to pH changes. Therefore, an RNA-removal step using RNase is always needed, which requires additional processes such as phenol/chloroform extraction.

Our new plasmid-purification protocol greatly improved on previous methods in two ways. First, we

modified Solution III (into Super Sol III) by adding calcium chloride to directly remove a large amount of RNA during the neutralization step. This allowed us to purify plasmid high quality DNA in fewer steps. It also did not require RNase incubation or a hazardous phenol/chloroform extraction step. Our modified Solution III removed unwanted RNA in the neutralization step without centrifugation. A small amount of RNA still remained in the cleared lysate, which was easily removed by simple polyethylene glycol precipitation. Second, we added a filtration step. Unlike an anion-exchange column and/or a silica membrane column, our column simply filtered and separated insoluble debris. In our protocol (Figure 4), centrifugation was performed before filtration not to precipitate RNA but simply to reduce debris. Use of a commercial filter and/or gel filtration resin (such as Sephadex) may lead to a much better result, although our handmade column was sufficient for our experiments. The syringe filter was easier to handle, although the total quantity was better in the handmade-column than the syringe (Table 1). This might be due to the dead volume of the syringe filter. The syringe filter is still applicable because the plasmid purified using the syringe filter had a quality good enough for downstream experiments. In our manuscript, Figure 2, Table 1 (in part) and Table 2 were data using the syringe filter, and others were from the handmade-column. We tested both filters and concluded that they worked well in our daily experiments (data not shown).

Too large of an amount of *E. coli* at the start (*i.e.* too much *E. coli* cells for reagent volumes) results in insoluble impurities in the final plasmid solution. It is important to keep a volume balance between solutions and *E. coli*. In large-scale experiments, simply dividing *E. coli* samples into several test tubes will give a good result. An option for scaling up is to use a 50-mL polypropylene centrifuge tubes and 15-mL polystyrene round-bottom tubes for the handmade-column, instead of the tubes indicated in Figure 1. We checked and confirmed that this scaled-up protocol worked well up to 50-mL LB medium (data not shown).

Generally, super-high-quality plasmid DNA is required for injection into *C. elegans* or transfection into cultured cells. Our data strongly suggests that plasmid DNA isolated by our protocol is of high enough quality for use in biochemical reactions and transformations. A Sigma commercial kit showed better transfection efficiency for cultured cells than our method, but it is very surprising that we can prepare a transfection-grade plasmid by such a simple protocol as described in this manuscript. This commercial kit describes that up to 15 μg of plasmid DNA can be purified from 1-5 mL of *E. coli* culture (10), which is a better quantity than our method. Nevertheless, our method has good quality and quantity for downstream experiments (Table 1). Besides, our method has significant advantages that we

do not need RNase, any special reagents or equipment. The column and syringe are recyclable, so that we do not need to take these costs into account.

Acknowledgements

We thank Ryo Kinoshita for valuable advice.

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(Received October 5, 2013; Revised October 30, 2013; Accepted November 3, 2013)