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ENHANCING YIELD OF LOW COPY NUMBER PLANT BASED VECTOR FROM *E.COLI* CELLS FOR GENE CLONING

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Abstract
Plasmid DNA is an important role play for gene cloning and gene expression analysis in *E.coli* cells. But some of the plasmids are very low copy number so its isolation is also difficult due to its low copy number. So in this study we used different types of media (broths) with more volume, area, specific growth temperature, time of incubation and quality of inoculum. We used five different types of broth for enhancing the yield of low copy number plasmids such as LB, LB24, SB, SOC and 2xYT media. In these five media the differences is low and high concentration of yeast extract and tryptone and in SOC media some different salts and glucose are additional. Comparison of these five media for enhancing the yield of low copy number plasmid DNA, SOC broth optical density is more comparative to other media. In SOC media plasmid culture OD and isolated plasmid DNA concentration both are directly proportional to each other. For enhancing the yield of low copy number plasmid DNA in *E.coli* cells these following factors are very important role play, quality of inoculum,size of inoculum specific incubation temperature, area of shaking vessel, incubation time, required concentration of antibiotics, and also concentration of tryptone, yeast extract and additional salts and glucose. One another important factor for enhancing the yield of low copy number plasmid used manual method (alkaline lysis method) for isolation of low copy number plasmid DNA and its purification also effects the yield of plasmid DNA.
Keywords: Enhancing Yields, Low Copy Number, Plasmid DNA, Media.

INTRODUCTION

For gene cloning, expression, sequencing, and mutagenesis, molecular biologists frequently employ circular plasmids grown in Escherichia coli cells. According to Sommers (1998), Feinbaum (2002), Nordstrom and Dasgupta (2006), these plasmids are commonly divided into three groups: high copy number, which corresponds to hundreds of plasmid DNA molecules per cell; low copy number, which is usually less than fifty plasmids per cell; and single copy number, which is roughly one per cell. For straightforward DNA fragment cloning, high copynumber plasmids are the most common vectors. These plasmids are extracted from E. The DNAs are often extracted from 1–5 mL of overnight cultures of bacterial cells (also known as plasmid minipreps), and coli cells yield high yields. In contrast, a large number of protein expression vectors are low copy number plasmids. A lower copy number (lower gene dosage) can lower basal, or uninduced, intracellular levels of an expressed protein. This is advantageous because some foreign proteins can inhibit *E.coli* cells growth because they are toxic even at very low levels. coli cells. IMPACT system vectors (New England Biolabs), HaloTag vectors (Promega), the popular pET series of vectors (Novagen), and a number of others (Dersch et al. 1994; Mardanov and associates. Cheong et al. (2007). (2013) Guan et al. 2013). To extract these plasmids, larger cell cultures must be inoculated because their yields are significantly lower than those of high copy number plasmids.

The most popular liquid medium for *E.coli* growth. LB (Lysogeny Broth) is the ideal broth for *E.coli* cells, but over time, a number of other broths have been created and used for particular procedures.

Some examples of these additional media are 2xYT (Yeast extract Tryptone), SB (Super Broth), SOC (Super Optimalbroth with Catabolite repression), and TB (Terrific Broth) (Tartoff and Hobbs, 1987; Elbing and Brent, 2002; Green and Sambrook, 2012; Lessard, 2013). To *E.coli*, SOC is added. coli cells in numerous chemical-based DNA transformation procedures to promote recovery following exposure to high salt concentrations and heat shock, and 2xYT is employed in numerous bacteriophage infection procedures (Elbing and Brent, 2002; Green and Sambrook, 2012). The amounts of tryptone and yeast extract in each broth vary, but these two nutrients are present in all of these growth media. Some of the broths contain particular extra substances, like glycerol, glucose, and/or salts, that are good for cell growth.

In this current study, we have tested five well established *E. coli* broths to determine whichone produces the highest yields of low copy and single copy number plasmid DNAs from1.5 mL minipreps. TB broth consistently produced the greatest DNA yields. The strongresults with TB were found to be independent of its glycerol content and, instead, wereprimarily due to its high concentration of yeast extract. Adding increasing amounts of yeastextract to LB broth produced higher DNA yields in a concentration-dependent manner. Theresults demonstrate that yields of both low and single copy number plasmids isolated usingstandard alkaline lysis minipreps can be strongly enhanced using simple, inexpensive mediacontaining elevated levels of yeast extract.

MATERIALS AND METHODS

Strains, Plasmids and Reagents

Prepare competent cell of *E.coli DH5a* by using $Cacl_2$ method and then transformed pCAMBIA 1304 vector in *E.coli DH5a* cells by using heat shock method. In *E.coli DH5a* competent cell add DNA and incubate on iceand then give heat shock and incubate at 37°C for two to three hours then plate LB agar with ampicillin 100ug/ml and then incubate overnight at 37°C. After overnight incubation the transformed colonies are formed, then pick this colony and subculture with freshly prepared five different broths with 50µg/ml concentration of kanamycinand incubate at 37°C for 24 hours. All broths were prepared according to the descriptions published in Current Protocols in Molecular Biology (Elbing and Brent, 2002). Recipes used to prepare 1 L of each broth were the following:

- LB: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 mL 1 M NaOH.
- LB24: 10 g tryptone, 24 g yeast extract, 5 g NaCl, 1 mL 1 M NaOH.
- SB: 32 g tryptone, 20 g yeast extract, 5 g NaCl, 5 mL 1 M NaOH.
- 2xYT: 16 g tryptone, 10 g yeast extract, 5 g NaCl.
- SOC: 20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 3.6 g glucose, 2.5 mMKCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

DNA Gel Electrophoresis and Cell Culture Density Determination

Cell culture densities were monitored by measuring light scattering at 600 nm using a shimadzu UV-1700 spectrophotometer after diluting overnight cultures 300 times into water. Electrophoretic analysis of purified DNAs employed Horizon (Biorad), 1 x TAE electrophoresis buffer and 0.8–0.9% agarose gels run at approximately 50 mA. Gels were stained with ethidium bromide and photographs were taken using an Gel Documentation System instrument (Biorad).

Plasmid DNA Isolation Protocols

The majority of plasmid minipreps were carried out using a standard alkaline lysis protocol (Elbing and Brent, 2002; Green and Sambrook, 2012) in conjunction with 25 mL cell cultures containing 100 µg/ml ampicillin that were shaken overnight (22–24 hours) at ~ 250 rpm at 37°C in 100 ml glass conical flask .. To summarize, standard alkaline lysis minipreps involved pelleting 1 ml*3 times cells in a microcentrifuge for 60 seconds, then adding Solution I (50 mM glucose, 25 mMTris [pH 8.0], 10 mM EDTA), fresh Solution II (0.2 M NaOH, 1 percent SDS), and cold Solution III (3 M KOAc). To reduce chromosomal DNA contamination, supernatants were moved to a new tube after proteins and cell debris were sedimented for 10 minutes at 15,000 rpm. Only the liquid that was about 4 or mm above the protein pellet was removed. After adding 0 point 7 volume isopropanol and centrifuging for 5 minutes, the DNA was precipitated. The DNA pellets were cleaned with 0–5 mL cold 70% ethanol, allowed to air dry in a laminar tube for 30 minutes, then resuspended in 50µL TE (10 mMTris [pH 8.0]), 1 mM EDTA,

and an equal volume of 5M lithium chloride. The supernatant was then transferred to a new tube, and the pellet was discarded. Two volumes of absolute ethanol were added to the supernatant, which was incubated on ice for 15 minutes and then at 15000 rpm for 15 minutes under cooling conditions. This process is used to remove high molecular weight RNA. The pellets were then air dried and resuspended in 50ul T10E1 buffer, and finally, 5ul RNase A from 10mg/ml.
For experiments used five different types of broths, all overnight cultures were inoculated with the same initial number of *E. coli* cells and shaken together at 37°C for 48 hours. Typically, these broths are inoculated with 1% log phase culture plus ampicillin 100ug/ml in each broth.Average results were calculated after DNA concentrations and cell densitiesand are shown in the figures 6 and 7.

Transformation Procedure for *E. coli DH5α*
Tube of competent cells was out of -80°C and was putted on ice for allowing it to thaw to 20min.100 µl of competent cells were transported to pre-chilled tube and remained cells were refrozen in -80°C. 5µl of plasmid DNA pCAMBIA1304 was adding to the competent cells and the contents were mixing gently by moving the pipette through cells, the competent cells/ plasmid mixture was incubated on ice for 30 min. Each transformed tubes were shock heated by placed the tube into water bath at 42°C for 45 sec. And then the tubes were placed immediately on ice for 2 min. 450µl of LB broth without antibiotic was added to the tube and incubated in a water bath shaker at 37°C with shacked at 200 rpm for 2 hour.100 µL of transformed bacteria were cultured on LB plates containing 50 µg/mL kanamycin. The control tube was making without added plasmid. The plates were incubated at 37°C overnight. The colonies appeared in the plates were counted and the transformation was confirming blue white screening if the plasmid have lac Z gene.

CONFIRMATION OF LOW COPY NUMBER PLASMIDDNA BY USING BLUE WHITE SCREENING (if plasmid have Lac Z gene)
Screening on Pre-made Agar Plates Lacking IPTG and X-Gal
1. Pour autoclaved growth media containing screening antibiotic on media plates and dry in a laminar flow hood. Blue White Screening using X-Gal and IPTG 2. Add 40 µl 100mM IPTG and 120 µl X-Gal (20 mg/ml) to the surface of each plate and spread over the entire surface. 3. Dry X-Gal/IPTG-coated media in a laminar flow hood for approximately 30 minutes before use. 4. Spread transformed competent cells and incubate inverted at either 37°C until blue colonies form (usually ~24 hours).

RESULTS AND DISCUSSION
TRANSFORMATION OF pCAMBIA 1304 VECTOR IN *E.COLI* CELLS AND CONFIRMED BY BLUE WHITE SCREENING

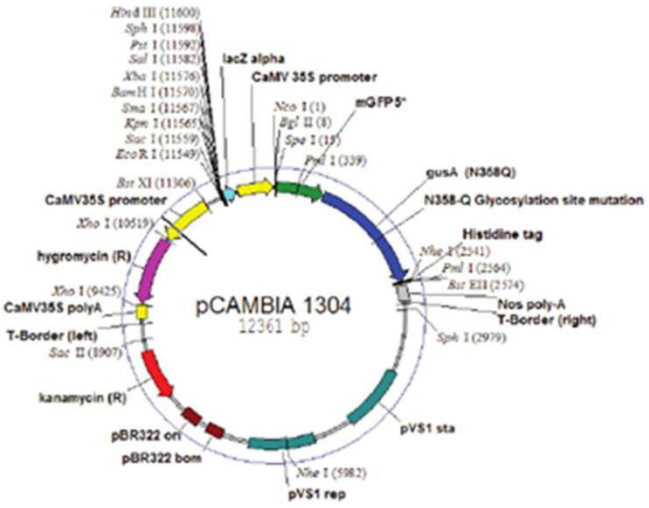

Fig.1. Vector map of pCAMBIA 1304

The transformed colonies is confirmed by blue white screening if the plasmid have LacZ gene and transformed into *E.coli DH5a* cells. The low copy number plasmid is also confirmed by using blue white if the plasmid have LacZ gene. After transformation if the transformed colonies were shown blue colour it means presence of transformed low copy number plasmid shown in Fig,3. with presence of IPTG and X-Gal without IPTG and X-Gal transformed colonies were not shown blue colour colonies.

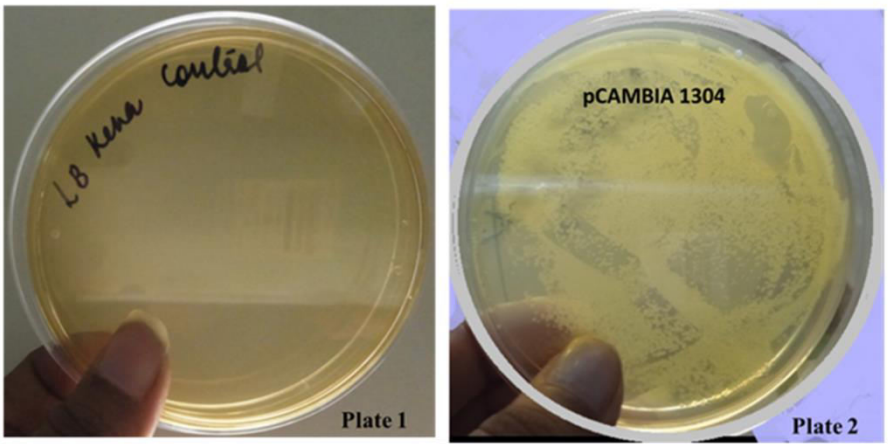

Fig.2. Transformed colonies of pCAMBIA 1304 in *E.coli DH5a* plate 1 is Control and plate 2 is pCAMBIA 1304

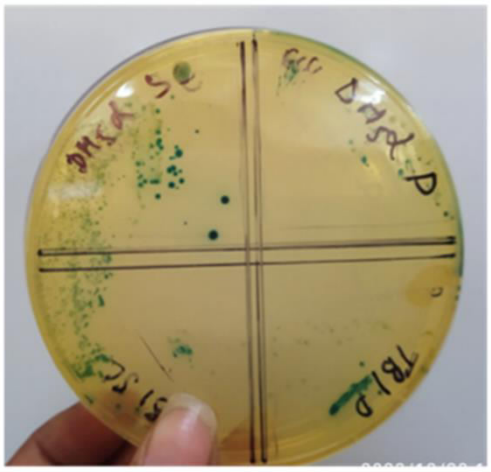

Fig.3. Confirmation of transformed low copy number pCAMBIA 1304 vector colonies by using blue white screening

OPTIMIZE YOUR BACTERIA

Sometimes particular *E. coli* strains are sub-optimal for plasmid extraction. If you are experiencing low yields for your plasmid prep, double check that the strain you're using is best for plasmid propagation. Some strains are more optimized for protein expression than for efficient DNA replication. Others have unwanted by products, such as carbohydrates or endonucleases, which can co-purify with plasmids. When possible, stick to tried and tested strains like *E. coli* DH5α which contain mutations to lack certain endonucleases and increase plasmid stability. Thus, these modified *E. coli* strains are used as work horses for molecular cloning and plasmid production.

USE OPTIMAL GROWTH CONDITIONS

Never inoculate culture straight from your bacterial stock. Always start with a single colony that is then grown as a starter culture. This ensures that your culture is derived from the same genotype and is not a mix of different colonies which may not have the same characteristics.

Also, be sure to double check your growth conditions. While many guides will recommend 12-16 hour cultures, every *E. coli* strain is slightly different in its growth rate and final density. Some require different temperatures, longer incubation times, faster shaking speeds, or more oxygenation.
For example, the maximum culture volume should not exceed 1/5 the total volume of the growth flask or alternatively, growth in baffled flasks can be used to increase aeration, and thus, culture growth. Also, take note of the shaking speed of the culture, with 200-250 rpm typical.
Additionally, total plasmid yield can vary depending on the type of culture broth used, typically Luria-Bertani broth (LB) or Terrific Broth (TB). Don't be afraid to experiment with your growth conditions to see which gives you the best plasmid yield.

EVALUATION OF PLASMID DNA YIELDS BY USING FIVE COMMON BROTHS

The primary goal of this project was to identify broths and/or growth conditions that enhance yields of low copy number plasmids from 1.5 mL minipreps. The specific problem that was addressed is illustrated in Fig. 4. DNAs were purified from 1.5 mL LB broth + kanamycin after 48 hour cultures of *E.coli DH5a* cells containing pCAMBIA 1304 (low copy number plasmids, respectively) and 3 µl out of 50 total µl was mixed with loading dye and run on a 0.9% agarose gel. The high copy number plasmid preps produced intense bands corresponding to the supercoiled and nicked open circular forms of the plasmid. In contrast, faint bands are present in the lanes corresponding to the low copy number plasmids, demonstrating that cells grown in LB broth generate poor yields with these types of DNAs.
The potential for other well known broths to improve yield of the low copy number plasmid DNA pCAMBIA 1304 was investigated next. Cell culture densities and plasmid DNA yields were measured for cultures shaken 48 at 37°C in LB, LB24, SB, , 2xYT and SOC broths containing 50ug/ml kanamycin. All broths were inoculated with the same number of cells. Super broth consistently produced both the

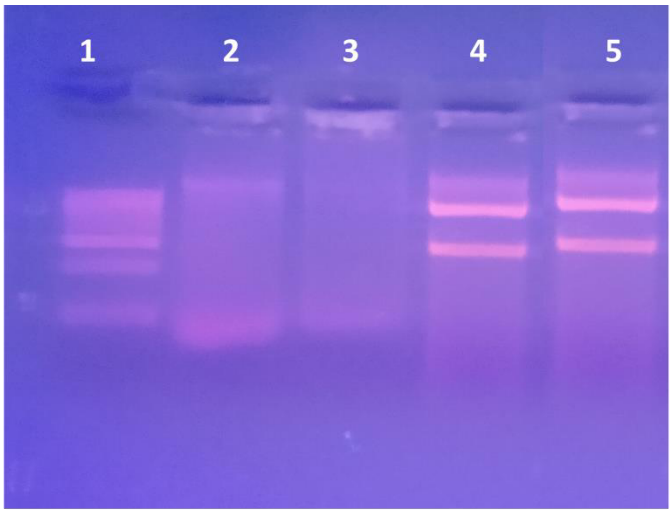

Fig.4. DNA Gel electrophoresis for use of the most common broth, LB, produces generous amounts of high copy number DNAs, but not low copy number plasmids, three µL of each 50 µL prep were loaded onto a 0.9% agarose gel. Results from two independent cultures are shown for each plasmid lane no. 2 and 3 for low copy number and lane no.4 and5 for high copy number

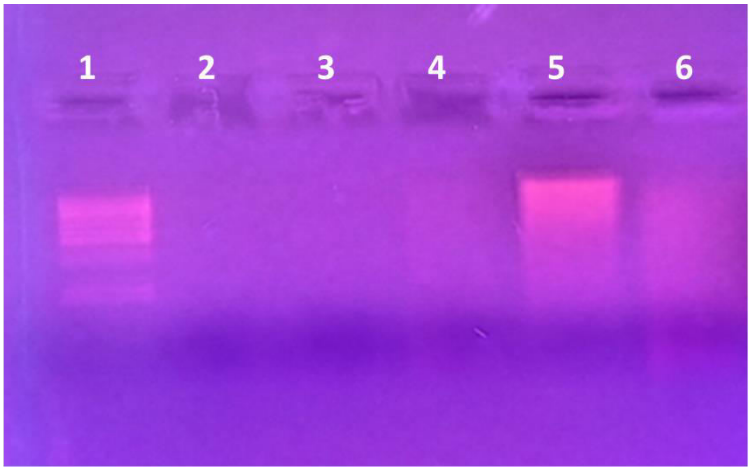

Fig.5. Enhancing yields of low copy number plasmid DNA (pCAMBIA1304) from *E.coli* cells by using different broth lane no. 1 1 kb DNA laddar, lane no. 2 LB broth, lane no. 3, LB24 lane no.4 Super broth, lane no.5, SOC broth and lane no.6 2xYT

highest cell densities (based on OD_{600} readings resulting from light scattering) and the highest DNA concentrations (Fig.6.and Fig. 7.). Cell densities achieved with SOC broth were over 3.1 times greater than those seen with the most commonly used broth, LB, and DNA yields were 8.53 fold higher (Fig.6.and Fig. 7.). This result was also apparent when an aliquot of each prep was run on an agarose gel, where SB, and 2xYT, LB broth, LB24 all outperformed (Fig.5.). SOC, the only broth containing supplemental glucose, consistently produced higher amount of growth of cells and also higher yield of DNA both the factors was enhanced concentration of cells and the concentration of DNA yield was enhanced compared to LB, LB24, SB and 2xYT (Fig.6.).

COMPARISION OF DIFFERENT AMOUNT OF YEAST EXTRACT IN FIVE DIFFERENT BROTHS AFFECTS CELL CULTURE DENSITIES AND PLASMID DNA YIELDS

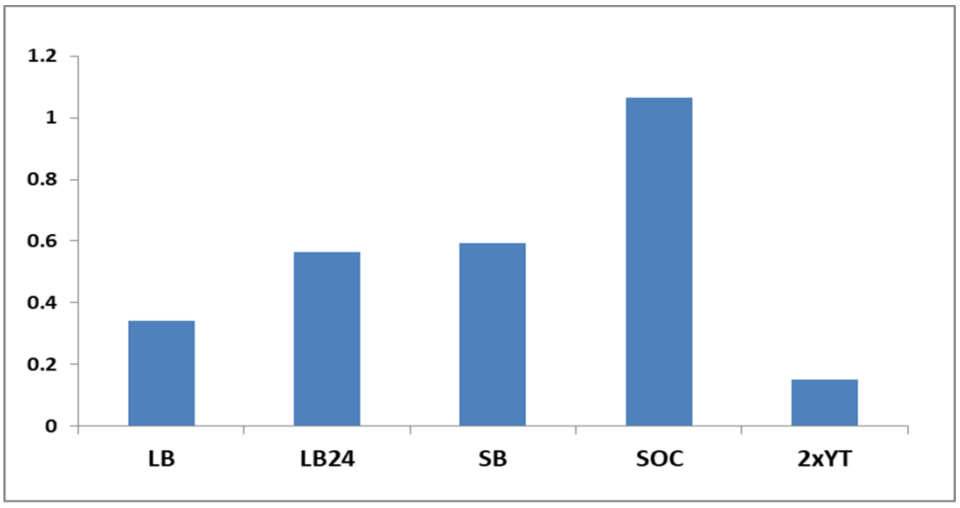

Fig. 6. Assessment of cell culture densities after using five different broths in LB, LB24, SB, SOC and 2xYT at x axis and optical densities at y axis. SOC broth cultures had the highest cell densities after shaking 48 hours at 37°C.

Another difference between LB , LB24, SB and 2xYT is the difference of yeast extract amount in these different broths. The impact of this nutrient was assessed initially by comparing DNA yields in

(a) LB 5g/L versus LB24 prepared with 24 g/L yeast extract (LB24), SB 20g/L, 2xYT 10g/L and SOC 5g/L. As shown in Fig.5,, while the amount of yeast extract in these broths are different. The growth yield of cells in these five different broths are different and also the concentration of DNA yield is different, the highest growth yield of cells was measure at OD_{600nm} that is seen in SOC strongly increased DNA recovery.

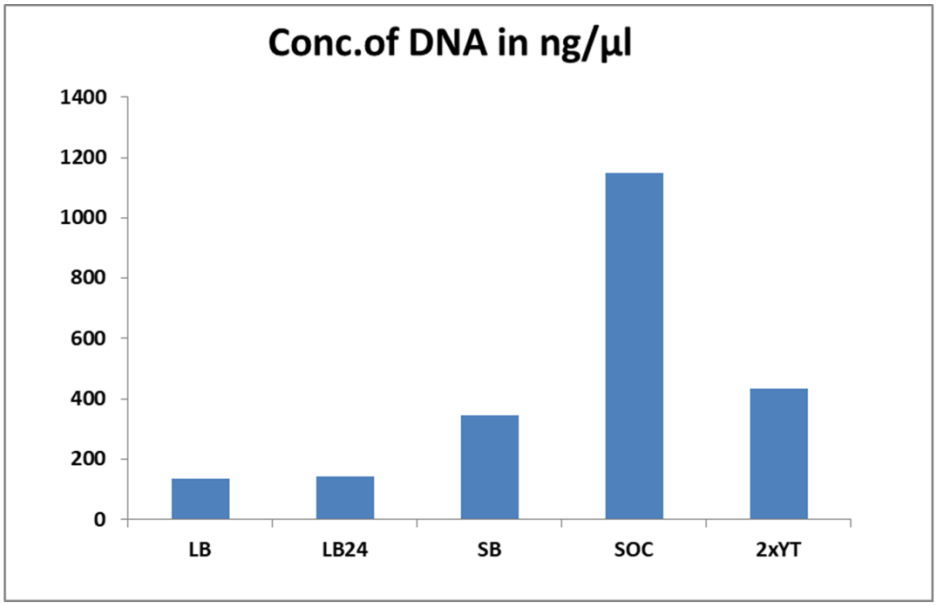

Fig. 7. Assessment of yield of DNA in ng/µl after propagation in five different broths LB, LB24, SB, SOC and 2xYT. (B) SOC broth cultures had the highest DNA yield after shaking 48 hours at 37°C

YEAST EXTRACT BROTHS PERMIT ISOLATION OF PRACTICAL AMOUNTS OF LOW COPY PLASMID DNA FROM MINIPREPS

Purification of low copy number plasmids results in even lower yields than low copy plasmids , and it is common to perform midipreps (i.e., using ~ 100 mL of cells or more) rather than minipreps to isolate these types of plasmids. We tested the ability of broth with a high level of yeast extract to enhance recovery of these plasmids from small cultures. *E.coli* cells containing pCAMBIA 1304 were grown 48 hours broths containing kanamycin as before. We have observed previously that minipreps performed using Thermo Fisher Scientific miniprep kits typically produce plasmid DNA preparations with a low amount of the nicked open circular form (upper band) visible on gels such that more molecules are concentrated into a single supercoiled DNA band. This is advantageous for visualization of small amounts of DNA after staining with ethidium bromide because all of the staining is concentrated into one intense band rather than being split into two weaker bands. Plasmid pCAMBIA 1304 DNAs purified from 1.5 mL overnight cultures using Thermo Fisher Scientific kits were analyzed by spectroscopy and gel electrophoresis. Yields of these DNAs were considerably lower than with the high copy number plasmids but, importantly, SOC generated around 8.53times as much DNA as LB broth (Fig.7.). Electrophoresis of DNAs isolated from different broth cultures confirmed the purity and yield enhancement in different yeast extract in different broths (Fig.5.). Note that 3µL of each 50 µL prep were loaded onto the gel shown in (Fig.5.), which is more than was used for the previous gels. Use of DNA stains with greater sensitivity with ethidium bromide would permit smaller volumes to be used for electrophoresis, thereby improving the analysis of low copy number plasmids even further.

CONCLUSION

In conclusion, we have analyzed several commonly used *E. coli* cell culture broths and determined that SOC produced the highest yields of low copy number plasmid DNA from minipreps and

midiprep. This result is consistent with some previous studies of protein expression in *E.coli* cells that noted improved yields of protein when TB was used (Li et al., 1990; Nakamura et al., 1999; Losen et al., 2004; David et al., 2015). SOC. (Super Optimal broth with Catabolite repression) Medium is used in the final step of bacterial cell transformation to obtain maximal transformation efficiency of *E. coli*. Catabolite repression is achieved by providing glucose in the medium and thus creating optimal metabolic conditions for *E. coli*. In addition, high nutrient concentration allows cells to recover after the stress induced by transformation and achieve 2–3 fold higher efficiency as compared to recovery in LB medium.The superiority of SOC medium to help transformed *E. coli* cells recover from the stress of pore formation, these cells need to live in the best growing environment. Special media, such as SOC medium, is a nutrient rich medium It contains all nutritional requirements and growth factors. This medium allows the transformed cells to recover optimally. In the SOC medium, tryptone and yeast extract provide *E. coli* with a source of carbon and nitrogen, in addition, the medium contains minerals, vitamins and amino acids. Sodium chloride and potassium chloride in the medium maintain ion transport and osmotic balance. Magnesium sulfate is a source of magnesium ions required for some enzymatic reactions. Glucose is an additional source of carbon and energy for metabolism, supporting faster recovery and growth of *E. coli*. This combination of salts, magnesium and glucose in the SOC medium stabilizes the transformed cells and supports plasmid uptake (Lessard, 2013). As a result, growing *E. coli* after transformation in this medium also increases the transformation efficiency.

This result demonstrates that complex broths such as SOC, and more exotic alternatives such as H15 which contain supplemental buffers, salts and additional carbon sources (Tartoff and Hobbs, 1987; Duttweiler et al., 1998; Lessard, 2013) are not needed to generate high plasmid DNA yields. Recovery of the low copy number plasmid pCAMBIA 1304 and its yield was high by using SOC media due to additional sources of minerals, vitamins and amino acids. Yields of these plasmids were quite low comparatively to high copy number plasmids.However, these quantities were more than sufficient for analysis by gel electrophoresis andare likely to be sufficient for many downstream applications. Finally, we note that yields of the low copy number plasmids could not be improved by increasing the concentration of antibiotic in the growth media, i.e., by exerting greater selective pressure for retention of the plasmids by the cells.

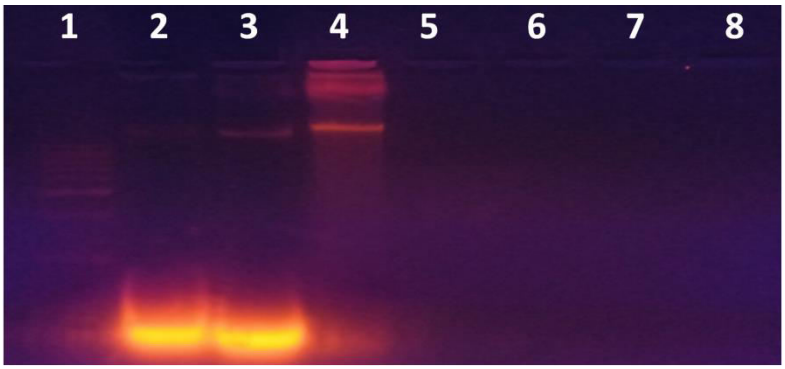

Fig.8. Electrophoresis of Isolation of low copy number plasmid DNA by using manual method (alkaline lysis method) lane no. 1kb DNA laddar, lane no.2 isolated plasmid without RNase treatment, lane no.3 Isolated plasmid after lithium chloride and lane no.4 Purified plasmid.

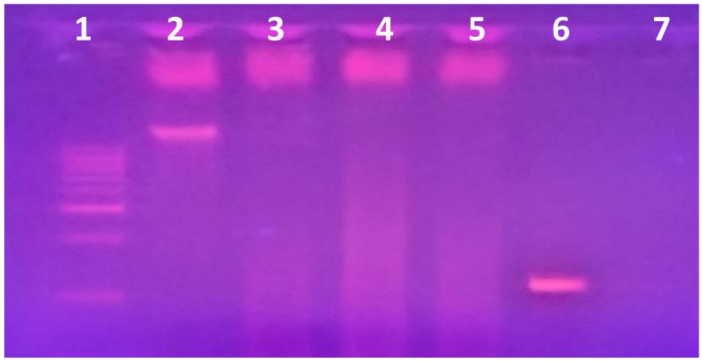

Fig.9. Restriction Digestion of low copy number plasmid DNA pCAMBIA 1304 in lane no.1 1 kb DNA laddar lane no.2 uncut plasmid DNA lane no.3 Single cut with XbaI size is 12361 bp lane no. 4 Double cut with XhoI size is 1094bp and 11267bp lane no.5 Tripple cut with MluI size is 18bp, 698bp and 11645bp

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