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Removal of RNA Contamination from Isolated Bacterial Plasmid DNA by Antichaotropic Salts

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Abstract: ***The demand for efficient production methods of plasmid DNA (pDNA) has increased vastly in response to rapid advances in the use of pDNA in gene therapy and in vaccines since the advantageous safety concerns associated with non-viral over viral vectors. Preparative-scale purification of plasmid DNA has been attempted by diverse methods, including precipitation with solvents, Salts,detergents and chromatography with ion-exchange, reversed-phase, and size-exclusion columns. Chromatographic methods such as hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC) and size exclusion chromatography (SEC) are the only effective means of eliminating the closely related relaxed and denatured forms of plasmid as well as endotoxin to acceptable levels. However, the anticipated costs of manufacturing-scale chromatography are high therefore as an alternative we suggest the use of high salt solution for primary precipitation of RNA in a pharmaceutical-grade plasmid DNA purification. Five antichaotropic salts were tested for their potential to precipitate RNA. Calcium chloride was by far the best precipitant with high RNA removal in a very short incubation time. Optimized conditions for calcium chloride precipitation were then introduced to the plasmid purification process resulting in the efficient removal of most impurities (RNA, chromosomal DNA and proteins).***
Keywords: Plasmid DNA, antichaotropic salts, precipitates RNA, plasmid purification.

I. INTRODUCTION

Until the advent of gene therapy, plasmid purification was essentially confined to small bench-scale operations in molecular biology laboratories (1). The challenge for the gene therapy industry is to design a downstream plasmid process that is scalable, robust, and meets purity, potency, identity, efficacy, and safety standards set by regulatory agencies (2). The plasmid product must be of high purity, essentially in its supercoiled form and free of host-cell proteins, chromosomal DNA, RNA, and endotoxins (3). It is generally believed that gene expression is conferred by plasmid in its supercoiled form, so other topologies - such as open circular, linear, or multimeric - should be kept to a minimum. Over the last 20 years, the initial thoughts of gene therapy have been transformed into reality with more than 175 clinical trials and 2,000 patients already treated (4). Yet with all the trials, there is still no conclusive evidence for efficacy. Although the expectations have exceeded the initial success of this relatively new field, important information has been gained from preclinical and clinical trials.

Nonviral vectors are less efficient at gene delivery but have the advantages of low immunogenicity, better safety profiles, and improved stability. Additionally these are easy to handle and cheaper to manufacture. One of such commonly used non viral vectors is plasmid based vector. However, the rise of variant Creutzfeldt–Jakob (vCJD) disease in the UK has meant bovine-derived products (such as lysozyme, proteinase K, and RNase A) are no longer recommended for pharmaceutical production intended for humans (5).

Selective precipitation of RNA removes the impurity while the molecule of interest (the plasmid) remains in solution. An antichaotropic salt at high concentration - such as lithium chloride (6), sodium acetate (7), ammonium acetate (8), ammonium sulfate (9), magnesium chloride (10), or calcium chloride (11) - is the most common RNA precipitant. Lithium chloride is the most widespread RNA precipitant used in molecular biology laboratories but is not recommended for the manufacture of pharmaceutical-grade plasmid DNA because of toxicity issues.

II. MATERIALS AND METHODS

E. coli DH5α cells were transformed by pUC18 plasmid (12) and transformed colonies were used for isolation of plasmid DNA. Plasmid DNA was isolated by standard alkaline lysis method (13).

The alkaline lysis method was repeated without use of lysozyme and RNase and the efficiency of this method was checked by comparing the results with the standard procedure (Modified Maxi Prep) and selective precipitation of RNA using antichaotropic Salts.(sodium acetate, ammonium acetate, magnesium chloride, lithium chloride and calcium chloride) as follows: -
100 µL of the isolated plasmid DNA was treated with 100 µl of the above salts of varying concentrations (1M to 6M) and was kept for precipitation under cooling conditions (around 10ºC) overnight. The above mixture was centrifuged at 15,000 rpm & 12,000 rpm to obtain supernatant and pellet respectively.OD_{260} and OD_{280} of both the supernatant and the pellet were taken and also both were run on 1% agarose gel. DNA and RNA bands were stained by ethidium bromide and also visualized on UV transilluminator and photographs were taken subsequently.

III. RESULTS

The present work was initiated with the transformation of bacterial strain (DH-5 α) devoid of plasmid with the pUC 18 plasmid; we found 500 colonies/µg of DNA was the transformation efficiency of the bacterial strain. Further the plasmid DNA was isolated by Maxi Prep and Modified prep plasmid preparation procedure and the successful isolation of plasmid DNA by both the methods was confirmed by gel electrophoresis. We precipitated RNA and purified the plasmids by treating plasmid preparations with the different concentrations of the salts with the incubation of 24 h at 4^0C. After the incubation the plasmids were separated from the precipitant (RNA) by centrifuging the contents at 12000 & 15000 (as stated) RPM. The purified plasmid preparations were checked for their total DNA concentration by OD_{260}/OD_{280} ratio.
Finally we have analyzed comparatively the effectiveness of the selected salts for the selective RNA precipitation where after plasmid preparation, we had subjected the plasmid DNA with the different concentrations for the selective removal of RNA of salts and we found calcium chloride, the best precipitant of the RNA with undetectable RNA contamination in supernatant (centrifuged at 12000 rpm for 20 min) which was confirmed by agarose gel electrophoresis as shown in the (fig. 2) We also did the comparative analysis of the best suited concentration of each salt for the effective RNA precipitation and removal. There also we found that calcium chloride is the best precipitant of the RNA as shown in fig. although other salt magnesium chloride also resulted in the better removal of RNA which can be considered as a second choice for the RNA removal next to calcium chloride.

IV. DISCUSSION

The plasmid isolated from the transformed cells showed the presence of RNA contamination which was precipitated by using 5 different RNA precipitant ,salts viz,sodium acetate,ammonium acetate,magnesium chloride, lithium chloride and calcium chloride. Sodium acetate proved to be a moderate RNA precipitant. It showed the presence of plasmid in the supernatant to a greater extent and maximum precipitated RNA was removed in the pellets.So, the centrifugation speed (15,000 RPM for 10 min) was appropriate.When ammonium acetate was used as RNA precipitant, plasmid was present in higher concentration in the supernatant than in pellet but RNA contamination was more in the supernatant than in pellet. From such observation it is obvious that either the salt was not that efficient in precipitating RNA or the centrifugation speed was not sufficient to pellet out all the precipitated RNA. Magnesium chloride provided the evidence of better RNA precipitation as higher concentration was found in the pellet than in supernatant. Also the supernatant showed the presence of higher concentration of plasmid DNA, thus the centrifugation speed (12,000 RPM for 10 min)was also appropriate to pellet out most the precipitate RNA. Lithium chloride was found to be a moderate precipitant of RNA as precipitated RNA was found in greater concentration in pellet than in supernatant but still much concentration of RNA was found in the supernatant as well and also plasmid was found in greater concentration in supernatant .So centrifugation speed (12,000 RPM for 10 min)was appropriate.Calcium chloride was found to be the best RNA precipitant as it showed the removal of RNA to an undetectable level and no RNA was detected in the supernatant whereas maximum RNA was detected in the pellet. But higher concentration of plasmid was also detected in pellet .So the centrifugation speed (12,000 RPM for 10 min)was high and needs to be reduced.
On doing the comparative analysis of the optimum concentration of each salt viz 5M sodium acetate, 6M ammonium acetate, 6M magnesium chloride 1M lithium chloride and 3M calcium chloride .It was found that calcium chloride was the best RNA precipitant with no detectable level of RNA in the supernatant .Magnesium chloride was found efficient to a greater extent and hence it can be preferred as RNA precipitant as a RNA precipitant . The method here used for the detection of RNA contamination is agarose gel electrophoresis which is not more suitable for detection of RNA contaminants thus it can be advised that further estimation of RNA contamination can be done by modified Orcinol estimation or the RNA can be further purified by the techniques like Tangential flow filteration or chromatographic methods such as hydrophobic interaction chromatography (HIC) reversed phase chromatography (RPC) and size exclusion chromatography (SEC)for complete removal of RNA.

V. CONCLUSION

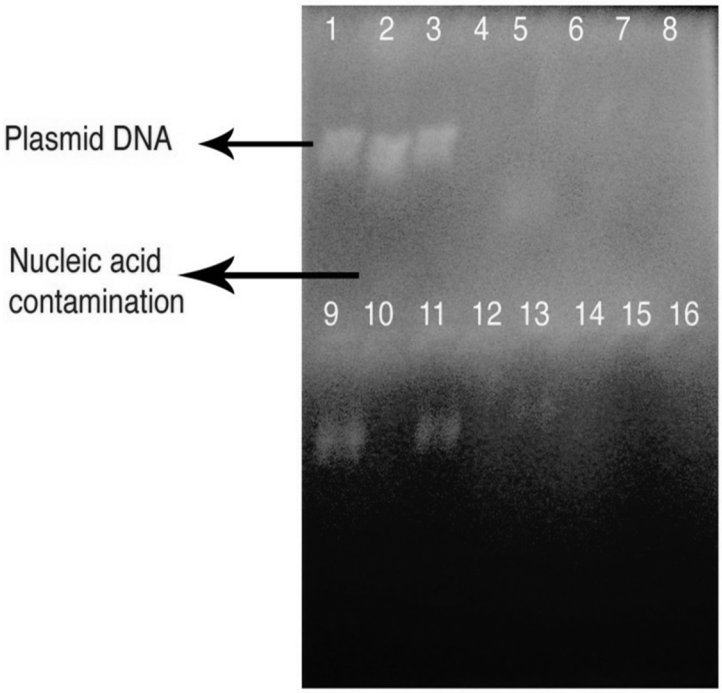
Comparative analysis of the optimum concentration of each salt viz. 5M sodium acetate, 6M ammonium acetate, 6M magnesium chloride, 1M lithium chloride and 3M calcium chloride. It was found that calcium chloride was the best RNA precipitant with no detectable level of RNA in the supernatant. Magnesium chloride was also found efficient to a greater extent and hence it can be preferred as RNA precipitant as a alternative RNA precipitant. The method here used for the detection of RNA contamination is agarose gel electrophoresis which is not more suitable for the detection of RNA contaminants thus it can be advised that further estimation of RNA contamination can be done by modified orcinol estimation or the RNA can be further purified by the techniques like Tangential Flow Filtration or chromatographic methods such as hydrophobic interaction chromatography (HIC), reversed phase chromatography (RPC) and size exclusion chromatography (SEC) for complete removal of RNA.

3. Observation table

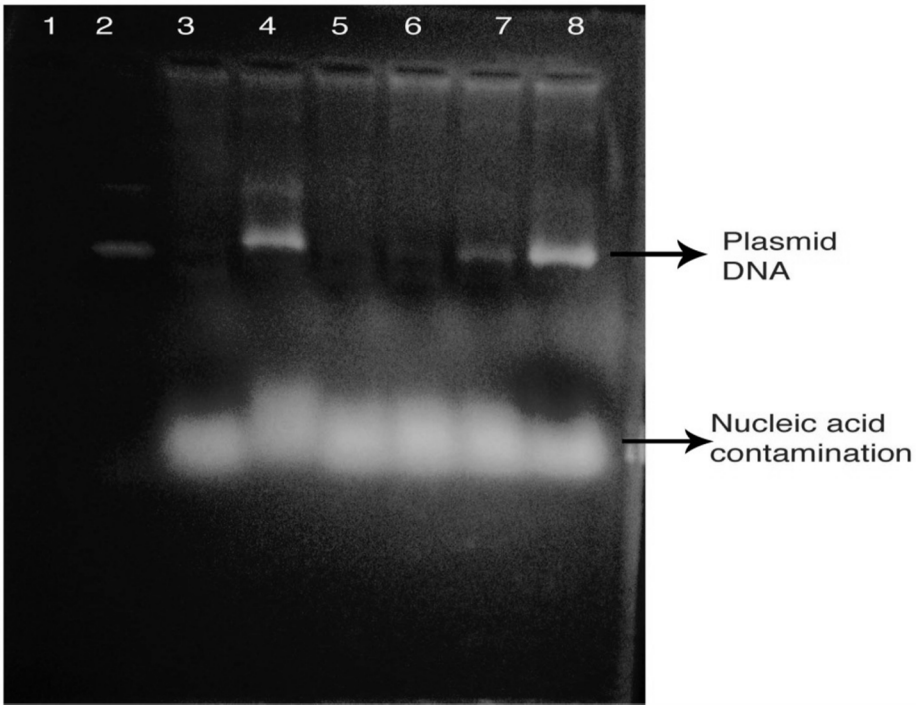
(RPM = 12,000 for 10 min at 4ºC)

Antichaotropic salt	Conc. of salt	OD_{260} (SUP)	OD_{260} (PELLET)	OD_{280} (SUP)	OD_{280} (PELLET)	Conc. of DNA in supernatant (µg/ml)	Conc. of DNA in pellet (µg/ml)
Lithium Chloride	1 M	0.059	0.035	0.037	0.032	295	175
	2 M	0.058	0.052	0.035	0.042	290	260
	3 M	0.053	0.055	0.032	0.056	265	275
	4 M	0.052	0.063	0.031	0.063	260	315
	5 M	0.051	0.071	0.032	0.053	255	355
	6 M	0.038	0.072	0.028	0.052	190	360
Sodium Acetate	1 M	0.066	0.023	0.043	0.014	330	115
	2 M	0.061	0.021	0.039	0.011	305	105
	3 M	0.068	0.034	0.039	0.020	340	170
	4 M	0.067	0.045	0.037	0.021	335	225
	5 M	0.065	0.048	0.038	0.022	325	240
	6 M	0.092	0.029	0.057	0.014	460	145
Ammonium Acetate	1 M	0.049	0.017	0.028	0.010	245	85
	2 M	0.045	0.037	0.025	0.019	225	185
	3 M	0.047	0.031	0.024	0.016	235	155
	4 M	0.046	0.050	0.023	0.025	230	250
	5 M	0.044	0.052	0.024	0.027	220	260
	6 M	0.042	0.050	0.023	0.025	210	250
Magnesium Chloride	1 M	0.042	0.024	0.031	0.020	210	120
	2 M	0.044	0.033	0.033	0.027	220	165
	3 M	0.041	0.027	0.030	0.025	205	135
	4 M	0.042	0.039	0.029	0.028	210	195
	5 M	0.042	0.031	0.029	0.029	210	155
	6 M	0.045	0.039	0.030	0.034	225	195
Calcium Chloride	1 M	0.045	0.074	0.029	0.050	225	370
	2 M	0.041	0.075	0.028	0.056	205	375
	3 M	0.038	0.080	0.025	0.055	190	400
	4 M	0.038	0.079	0.026	0.056	190	395
	5 M	0.041	0.101	0.027	0.070	205	505
	6 M	0.044	0.079	0.028	0.067	220	395

Supernatant Figure 1.1



1. PUC18 plasmid DNA, 2.Plasmid without lysozyme & with RNase treatment 3. Plasmid with lysozyme & RNase treatment, 5. 1M, 7. 2M, 9.3M, 11. 4M, 13. 5M, 15. 6M



Pellet Figure 2.2

2. pUC18 plasmid DNA, 3.1M
4. 6M, 5. 2M, 6.3M, 7.4M, 8.5M

Fig.1:- Agarose Gel Electrophoresis showing plasmid banding pattern in Supernatant and Pellet after precipitation.

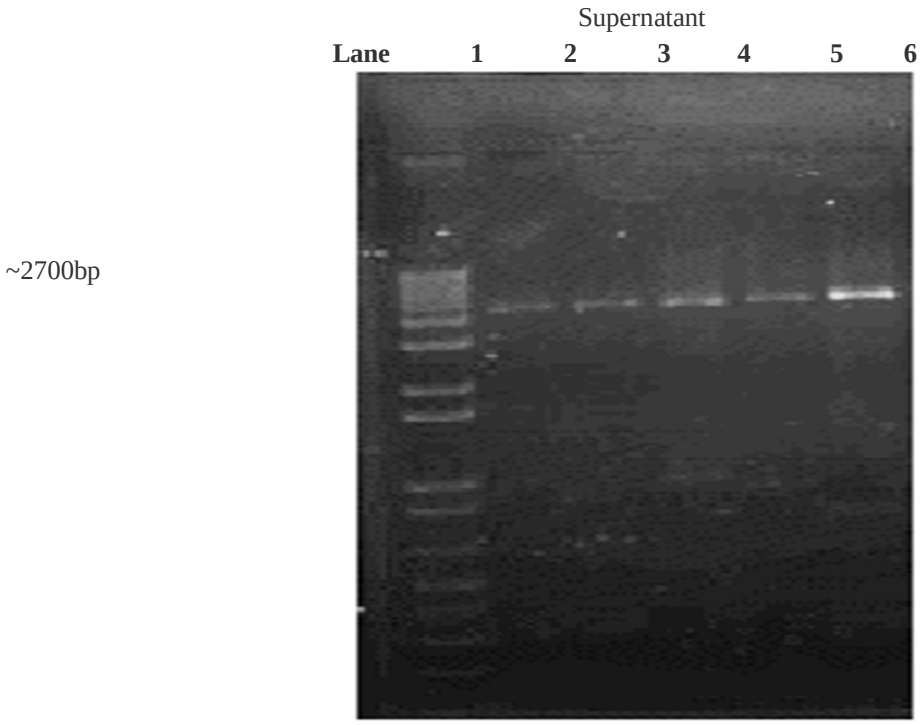
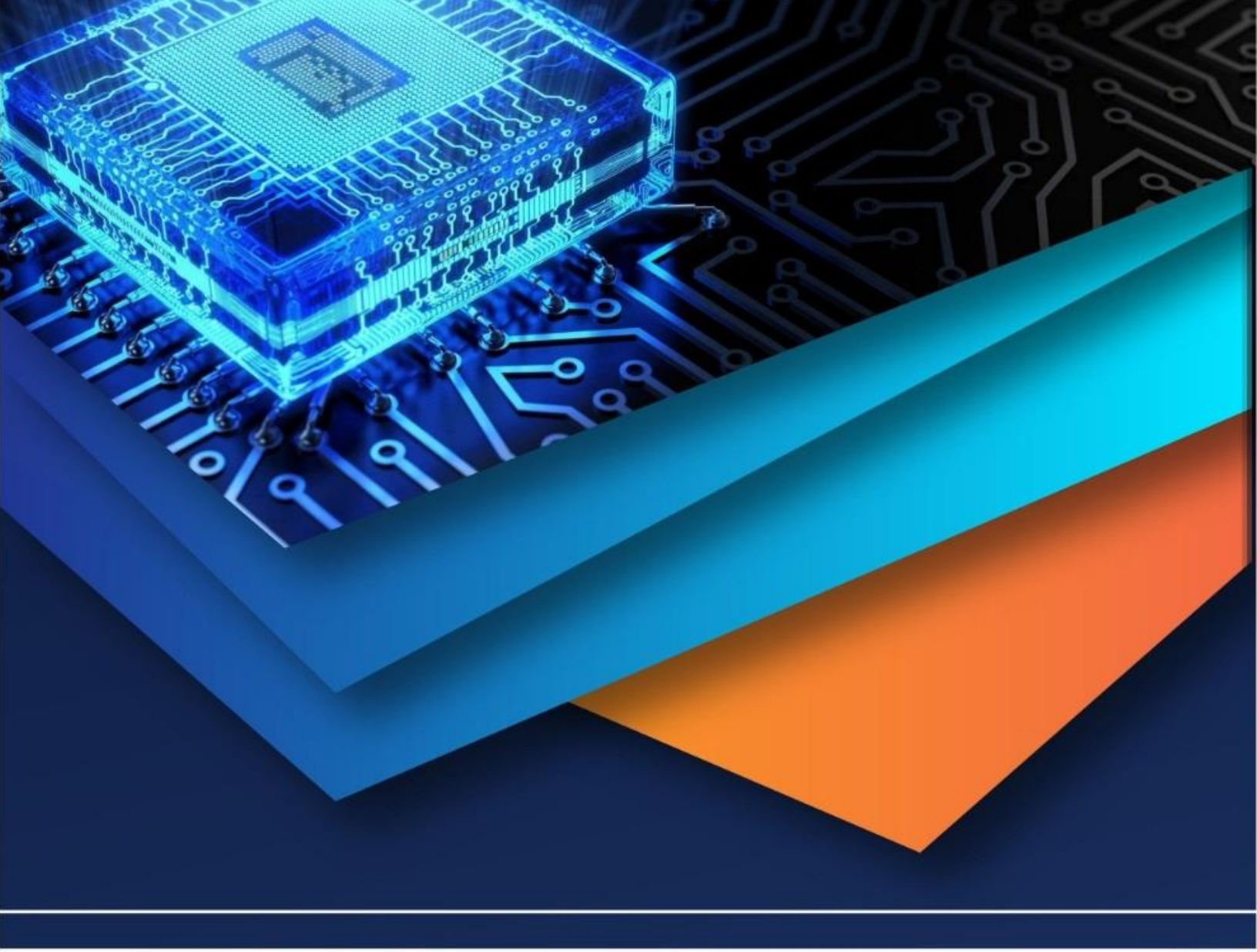


Figure 2: - Agarose Gel Electrophoresis showing comparative analysis of all antichaotropic salt precipitated plasmid DNA bands in Supernatant

1. DNA Ladder, 2. 5M Sodium acetate,
3. 6M Ammonium acetate, 4. 6 M Magnesium chlorides, 5. 1M Lithium chloride,
6. 3M Calcium chloride
(4000bp-100bp) DNA Ladder
4000bp,3000bp,2500bp,2300bp,2000bp,1500bp,1000bp,850bp,600bp,500bp,400bp,300bp,
200bp, 100bp, 50bp, 20bp.

REFERENCES

[1] Marquet, M., Horn, N.A., and Meek, J.A., "Process Development for the Manufacture of Plasmid DNA Vectors for Use in Gene Therapy," BioPharm 8(7), 26-37 (September 1995).
[2] CBER,Guidance for Human Somatic Cell Therapy and Gene Therapy (FDA, Rockville, MD, 1998).
[3] Prazeres, D.M.F. *et al*., "Large-Scale Production of Pharmaceutical-Grade Plasmid DNA for Gene Therapy: Problems and Bottlenecks," Trends Biotechnol. 17, 169-174 (1999).
[4] Ross, G., Erickson, R., Knorr, R., Motulsky, A. G., Parkman, R., Samulski, J., Straus, S. E. & Smith,B. R. (1996) *Hum. Gene Ther.* 7, 1781-1790 [ISI][Medline]Skovgaard, O., "Selective Precipitation of RNA with Mg2+ Improves the Purification of Plasmid DNA," Trends Genet. 6,140 (1990).
[5] Cooke, G.D. *et al.*, "Purification of Essentially RNA-Free Plasmid DNA Using a Modified Escherichia Coli Host Strain Expressing Ribonuclease A," J. Biotechnol. 85, 297-304 (2001).
[6] Wallace, D.M., "Precipitation of Nucleic Acids," Methods Enzymol. 152, 41-48 (1987).
[7] Evans, J.K. *et al.*, "Simultaneous Purification of RNA and DNA from Liver Using Sodium Acetate Precipitation," BioTechniques 24, 416-418 (1998).
[8] Green, A.P. *et al.*, "Preparative Purification of Supercoiled Plasmid DNA for Therapeutic Applications," BioPharm 10, 52-62 (1997).
[9] Sparks, R.B. and Elder, J.H., "A Simple and Rapid Procedure for the Purification of Plasmid DNA Using Reverse–Phase C18 Silica Beads," Anal. Biochem. 135, 345-348 (1983).
[10] Ugarov, V.I. *et al.*, "Plasmid Purification Using Hot Mg2+ Treatment and No RNase," BioTechniques 26, 194-198 (1999).
[11] Mukhopadhyay, M., and Mandal, N.C., "A Simple Procedure for Large-Scale Preparation of Pure Plasmid DNA Free from Chromosomal DNA from Bacteria," Anal. Biochem. 133, 265-270(1983).
[12] Calvin, N.M. and Hanawalt, P.C. High-efficiency transformation of bacterial cells by electroporation. *J. Bacteriol*. 170:2796-2801(1988.)
[13] Birnboim, H.C. "A Rapid Alkaline Extraction Method for the Isolation of Plasmid DNA," Meth. Enzymol. 100, 243-255 (1983)



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