© 1997 Oxford University Press

Nucleic Acids Research, 1997, Vol. 25, No. 19 **3959–3961**

Marked improvement of PAC and BAC cloning is achieved using electroelution of pulsed-field gel-separated partial digests of genomic DNA

Scott J. Strong, Yuko Ohta[1], Gary W. Litman and Chris T. Amemiya[2,*]

Department of Pediatrics, University of South Florida, All Children's Hospital, 801 Sixth Street South, St Petersburg, FL 33701, USA, [1]Department of Microbiology and Immunology, University of Miami School of Medicine, PO Box 016960 (R-138), Miami, FL 33101, USA and [2]Center for Human Genetics, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA

Received April 22, 1997; Revised and Accepted August 13, 1997

ABSTRACT

We describe a simple electroelution method for purifying large, gel-fractionated DNA molecules that alleviates the need for melting of the agarose and subsequent enzymatic agarose digestion. The method yields DNA that is visibly more intact than that purified from a standard agarose-digestion protocol and is more amenable to large-fragment cloning with PAC and BAC vectors. These findings are notable in that PAC and BAC library construction is a very labor-intensive and costly procedure, such that any net improvement in cloning efficiency is highly advantageous. This method also should prove useful towards other applications which require purification of very large DNA molecules, such as YAC cloning.

Successful cloning of large DNA fragments (100–300 kb) using the PAC (P1 artificial chromosome; 1) and BAC (F-factor bacterial artificial chromosome; 2) systems is critically dependent on the integrity of the insert DNA. This integrity is often compromised by heating (melting) and subsequent agarose digestion of the gel-purified partial digests (3,4). Although several investigators include sodium chloride (25–100 mM) to reduce this heat-induced DNA damage (3,5,6), we find that the relative quality of large DNAs (i.e., >75 kb) is markedly improved by electroelution into dialysis bags, thereby eliminating the heating step. This method results in higher efficiencies of PAC and BAC cloning in direct comparison to standard agarose-digestion methods.

Fractionation of *Mbo*I (or *Sau*3A) partial digests via preparative pulsed-field gel electrophoresis (PFGE) is performed essentially as described (1,6). After PFGE, the outer tracks containing the molecular weight standards and a small representative (edge) portion of the partial digests are cut off and stained with ethidium bromide. Successive fractions (3–5 mm) containing the desired size range of partially-digested DNA are then excised from the middle (unstained) portion of the gel.

Dialysis tubing (Spectrapor 2) is pretreated by heating membranes (8–10 cm strips) at 90°C in 1 mM EDTA/2% $NaHCO_3$ for 10 min, boiling in H_2O for 10 min, rinsing several times in H_2O, and stored at 4°C in 50% ethanol. Immediately prior to use, the membranes are rinsed thoroughly in sterile H_2O and then in sterile 1× TAE buffer (40 mM Tris–acetate/1 mM EDTA, pH 8). For electroelution (7,8), preparative fractions containing ~300 mg of gel (~300 µl; e.g., 10 × 6 × 5 mm) are equilibrated in 50 ml of sterile 1× TAE buffer at 4°C with occasional mixing for 30 min. A gel slice is placed lengthwise into the dialysis bag, one end is sealed with a dialysis clip, and sterile 1× TAE buffer (300–400 µl) is added. Air is removed carefully from the bag, the other end is sealed with a dialysis clip, and excess dialysis membrane is trimmed away. The gel slice is then positioned longitudinally to one side of the dialysis bag, i.e., parallel to one of the creased edges of the bag. A gel electrophoresis unit filled with sterile 1× TAE buffer is equilibrated at 4°C (cold room). The sealed dialysis bag is submerged completely in the gel electrophoresis chamber such that the length of the gel slice is parallel to the electrodes and the side containing the gel slice is closest to the negative pole. Electroelution is carried out using a field strength of ~4–5 V/cm and after 2 h the polarity is reversed for exactly 1 min to disassociate DNA that has impacted on the side of the membrane. The assembly is removed carefully from the buffer chamber, blotted dry, one of the dialysis clips is undone, and the DNA is removed gently using a wide-bore pipet tip. Eluted DNAs can be used immediately for PAC or BAC ligations without the need for further purification, however, it is recommended that the samples be quantified prior to setting up ligations (6).

Figure 1 shows an ethidium bromide-stained pulsed-field gel of differentially-treated samples containing three partial digest *Mbo*I fractions from the skate, *Raja eglanteria*. For each fraction, approximately equivalent amounts of DNA were: left untreated (U); electroeluted (E) by the protocol described herein; treated by melting (M) the gel slice at 72°C for 20 min in 1× TEN buffer (10 mM Tris–HCl/1 mM EDTA/50 mM NaCl, pH 8); or treated by melting the agarose as above and digesting with GELASE™

* To whom correspondence should be addressed. Tel: +1 617 638 7078; Fax: +1 617 638 7042; Email: camemiya@bu.edu

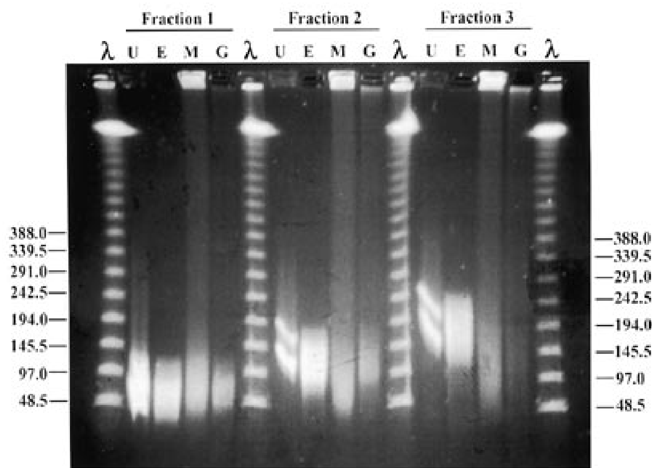

Figure 1. Analytical pulsed-field gel of *R.eglanteria Mbo*I partial digest fractions. Samples were sliced from individual fractions of a preparative gel and are: untreated (U), electroeluted (E), melted (M), GELASE^{TM}-treated (G). The 1% agarose gel was electrophoresed in a CHEF DRII unit (Bio-Rad) in 0.5× TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8) at 14°C, using a 10–60 s switch time at 6 V/cm for 18 h. Lambda concatemer size markers (λ) are indicated in kb. The average molecular sizes of the respective fractions determined by PAC cloning are: fraction 1, 50–75 kb; fraction 2, 75–100 kb; fraction 3, 100–150 kb (data not shown). These size ranges are not exactly those observed on the gel due to slight overloading of the samples needed to enhance visualization of the smaller-sized material and to PAC cloning/electroporation biases towards smaller clones. The doublet bands in 2U and 3U represent a gel loading aberration. For this experiment and subsequent ligation-transformation experiments, DNA-containing gel slices were melted for 20 min at 72°C in 1× TEN buffer and equilibrated for 15 min at 45°C, 1 U of GELASE^{TM} was added per 200 µl of respective sample and allowed to incubate at 45°C for 1–2 h; for cloning experiments, samples were subsequently drop-dialyzed for 3 h against 1×TAE buffer. It is important to note that the observed DNA degradation effect with melting and GELASE^{TM} treatment is not unique to this particular enzyme and also has been observed with agarose-digesting enzymes from at least two other manufacturers, as well as between batches of enzyme supplied from the same manufacturer (unpublished observations).

(Epicentre Technologies) (G). These results demonstrate that melting alone of agarose-containing partial digests gives noticeable degradation of the DNA (e.g., 2U versus 2M, 3U versus 3M). The inclusion of GELASE^{TM} accentuates the degradation effect and appears most severe for the two larger partial digest fractions (2G, 3G). By comparison, the electroeluted samples (1E, 2E, 3E) show more focused (less diffuse) distributions than the heat-treated and agarose-digested DNA samples, suggesting better integrity.

In order to test the relative cloning efficiencies of electroeluted versus agarose-digested DNAs, three ligation-transformation experiments were carried out: PAC cloning was performed for the three *R.eglanteria* partial digests shown in Figure 1; and both PAC and BAC cloning was performed using three successive size fractions of *Xenopus laevis-gilli* (*LG-15*) *Mbo*I partial digests within approximately the same size ranges as the *Raja* samples (i.e., 50–75, 75–100 and 100–150 kb). Vectors used for these experiments were: pCYPAC6 (unpublished modification of pCYPAC2; 6); and pClasper, a BAC shuttle vector with replicons for both yeast and *Escherichia coli* (9). Both vectors were linearized with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim), as described (1,6). Gel-separated partial digest fractions were purified in parallel using either electroelution or GELASE^{TM} digestion, and 20 ng of the recovered DNAs were combined with 20 ng of pCYPAC6 or 10 ng of pClasper in 20 µl ligation reactions (6). Conditions for ligation, drop-dialysis, and electrotransformation of *E.coli* cells have been described (1,6). Prior to transformation, all samples were adjusted to 20 µl to compensate for volume changes during dialysis. Negative controls (vector-only ligations) were run in parallel. One µl of each dialyzed ligation was added to 15 µl of DH10B^{TM} cells (GIBCO/BRL) and electroporated at a field strength of 16.6 kV/cm; cells were transferred to 1 ml of SOC medium (GIBCO/BRL) and expressed for 1 h at 37°C (300 r.p.m.), prior to spreading 200 µl of the suspension per 100 mm LB-agar Petri plate. pCYPAC6 transformants were spread onto plates containing kanamycin (35 µg/ml) whereas pClasper transformants were spread onto plates containing chloramphenicol (15 µg/ml). In order to statistically assess the repeatability and variance within and among treatment groups, a hierarchical experimental design was invoked that consisted of: two treatments (E and G), three partial digest fractions per treatment, two ligations per fraction, two transformations per ligation and four platings per transformation.

The data from the three cloning experiments are summarized in Table 1. Student *t*-tests (10) were used to compare treatment means to test the null hypothesis that there are no differences between treatments. The analyses show that the respective means for all fraction 2 and fraction 3 transformations are significantly different between treatments ($P < 0.01$), i.e., the electroeluted samples are superior over GELASE^{TM}-treated samples in terms of cloning efficiency regardless of insert DNA source or vector used. For fraction 1 of the *Raja* and *LG-15* pCYPAC6 experiments, no significant differences are observed between treatment means. A significant difference ($P < 0.05$) is observed for fraction 1 of the *LG-15* pClasper experiments, in which case the cloning efficiency of GELASE^{TM}-treated samples exceeds that of the electroeluted samples. This latter case notwithstanding, and more to the issue of the desired (larger) insert sizes, the trend in these analyses is that electroeluted DNAs yield greater numbers of transformants than the GELASE^{TM}-treated samples, particularly with the larger-sized fractions.

Lastly, in order to determine whether the different elution methods had any effect on insert sizes, 24 randomly-selected transformants from the *Raja* PAC cloning experiments were picked from each treatment (E or G) for each fraction (1, 2 or 3), and their insert sizes empirically-determined. Table 2 summarizes these results and clearly shows that the reduction in numbers of transformants in GELASE^{TM}-treated (versus electroeluted) samples for fractions 2 and 3 is not accompanied by any differences in insert size *per se*, but by a lower cloning fidelity (i.e., lower number of insert-containing clones). These findings support our claim that electroelution yields HMW DNA of higher quality and which is better suited for cloning.

In summary, we have described a simple alternative to enzymatic, agarose-digestion methods for purifying pulsed-field gel-separated partial restriction digests, that yields DNAs which are comparatively less degraded and more amenable to cloning. We and others (P.J.de Jong, personal communication) have successfully used this method for constructing genomic PAC/BAC libraries from numerous vertebrate species. Electroelution also should prove useful for yeast artificial chromosome (YAC) cloning and for purifying PFGE-separated YACs for mammalian cell transfection experiments.

Table 1. Summary of three experiments to test the efficiency of large-insert cloning for electroeluted versus GELASE™-treated partial digest fractions

Experiment[a]	Treatment	Fraction 1 (50–75 kb)				Fraction 2 (75–100 kb)				Fraction 3 (100–150 kb)			
		N	Mean	S.E.	*t*-test	*N*	Mean	S.E.	*t*-test	*N*	Mean	S.E.	*t*-test
Raja (pCYPAC6)	Electroelution	16	614.4	28.3		16	264.6	14.1		16	132.6	2.6	
	GELASE™	16	544.8	28.0		16	195.6	9.3		16	36.9	2.9	
					1.75				4.09*				24.61*
LG-15 (pCYPAC6)	Electroelution	16	633.3	36.6		16	172.4	4.2		16	102.6	5.0	
	GELASE™	16	607.0	11.7		16	72.1	2.6		16	10.4	0.6	
					0.68				20.21*				18.34*
LG-15 (pClasper)	Electroelution	16	665.6	14.5		16	222.2	7.6		16	65.0	2.3	
	GELASE™	16	733.3	20.7		16	85.4	2.8		16	11.0	1.1	
					2.68**				16.84*				21.29*

Each experiment is further subdivided into the respective fractions used for cloning (see text for details). The sample size ($N = 16$) represents pooled measurements of all platings (per transformation per ligation per fraction per treatment). Means are expressed as the average number of colonies per transformation and S.E. denotes standard errors of the means. Student *t*-tests were performed on the treatment means for respective fractions.

[a]Note, negative (vector-only) controls yielded ~10–25 colonies per transformation.

*$P < 0.01$.

**$P < 0.05$.

Table 2. Cloning fidelity and insert sizes of transformants from an experiment using *Raja* PAC ligations of fractions 1–3 from electroeluted versus GELASE™ treatments

Treatment	Fraction	Number of clones with inserts, *n*, out of $N = 24$ (%)	Average size of inserts per *n* (kb)	Average size of inserts per *N* (kb)
Electroelution	1	24 (100)	62	62
GELASE™	1	23 (96)	61	58
Electroelution	2	22 (92)	87	80
GELASE™	2	16 (67)	81	54
Electroelution	3	19 (79)	110	87
GELASE™	3	7 (29)	94	27

Representative clones from the *Raja* PAC cloning experiment in Table 1 were sized by *Not*I digestion of miniprep DNA and PFGE.

ACKNOWLEDGEMENTS

We thank Mr Asaf Halevi and Dr Tatsuya Ota (Boston University School of Medicine) for technical help and suggestions on the manuscript, Drs Suzanne Bradshaw and Frank Ruddle (Yale University) for the pClasper vector, Dr Louis Du Pasquier (Basel Institute for Immunology) for the *X.laevis-gilli* (*LG-15*) agarose-embedded DNA and Dr Jonathan Rast (Caltech) for suggesting (and help in) the initial electroelution experiments. This work was funded, in part, by the Charles H.Hood Foundation (Child Health Research Grant to C.T.A.), the National Science Foundation (grant IBN 9614940 to C.T.A.) and NIH (R29-AI39008 to C.T.A.; R37-AI23338 and R03-AI40137 to G.W.L.).

REFERENCES

1 Ioannou,P.A., Amemiya,C.T., Garnes,J., Kroisel,P.M., Shizuya,H., Chen,C., Batzer,M.A. and de Jong,P.J. (1994) *Nature Genet*., **6**, 84–89.
2 Shizuya,H., Birren,B.W., Kim,U.-J., Mancino,V., Slepak,T., Tachiiri,Y. and Simon,M. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8794–8797.
3 McCormick,M.K., Antonarakis,S. and Hieter,P. (1990) *GATA*, **7**, 114–118.
4 Larin,Z., Monaco,A.P. and Lehrach,H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4123–4127.
5 Riley,J. Ogilvie,D. and Anand,R. (1992) In Anand, R. (ed), *Techniques for the Analysis of Complex Genomes.* Academic Press, New York, pp. 59–79.
6 Amemiya,C.T., Ota,T. and Litman,G.W. (1996) In Birren,B. and Lai,E. (eds), *Nonmammalian Genome Analysis: A Practical Approach*. Academic Press, New York, pp. 223–256.
7 McDonnell,M.W., Simon,M.N. and Studier,F.W. (1977) *J. Mol. Biol*., **110**, 119–146.
8 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
9 Bradshaw,M.S., Bollekens,J.A. and Ruddle,F.H. (1995) *Nucleic Acids Res*., **23**, 4850–4856.
10 Cody,R.P. and Smith,J.K. (1991) *Applied Statistics and the SAS Programming Language*, 3rd edn, Elsevier Science Publications, New York, NY.