Comparison of Two Gel Electrophoresis-based DNA Micropurification Methods for Molecular Cloning

✉ Eugenio Hardy, Ricardo Silva, Elder Pupo, Lisset Hermida, Edelgis Coizeau, Olivia Niebla, Lila Castellanos-Serra

Center for Genetic Engineering and Biotechnology. PO Box 6162, Havana 10600, Telf.: (53-7) 21 6167; Fax: (53-7) 21 8070, 33 6008; E-mail: ehardy@cigb.edu.cu

ABSTRACT

We recently described a method for recovering nucleic acids separated from polyacrylamide gels, which is based on the sensitive on-gel DNA detection with zinc-imidazole followed by passive elution from 32-μm average-size gel microparticles into the elution buffer. The rationale for the preferential use of DNA micropurified by this method, as compared to DNA obtained by a conventional method—based on electrophoresis on agarose gels followed by ethidium bromide staining and UV light visualization—was evaluated. We found that micropurification of two DNA fragments, a 1.2 kbp DNA encoding the kanamycine resistant gene and the pUC19 vector, by both methods produced the same, statistically undistinguishable ($P = 0.10$) number of colonies when used to transform *Escherichia coli* XL-1 Blue competent cells. However, the number of transformants obtained in the absence of the insert was significantly lower when the DNA vector was micropurified from the polyacrylamide gel by the zinc-imidazole method ($P = 0.0002$), which increased the probability of finding desired recombinant colonies in the cloning experiment. These results encouraged the use of our zinc-imidazole–based technique for high quality micropurification of DNA and to overcome situations where DNA purified from conventional LGT agarose (e.g., genes for the class 1 outer membrane protein—both intact or with a deletion in loop 5—and Opc-protein from *Neisseria meningitidis* 866 strain) failed to give rise to recombinant *E. coli* cells.

Keywords: cloning, ethidium bromide, gel electrophoresis, ultraviolet-light, zinc staining

Biotecnología Aplicada 2001;18:29-31

RESUMEN

Comparación de dos métodos de micropurificación de ADN para clonación. Recientemente se describió un método para la recuperación de ácidos nucléicos separados en geles de poliacrilamida, el cual se basa en la detección sensible de ADN en el gel mediante zinc-imidazol seguido de la elución pasiva del mismo desde micropartículas de gel de 32 μm de tamaño promedio hacia el tampón de elución. En este trabajo se evalúa la racionalidad del uso preferencial de ADN micropurificado mediante este método, en comparación con el uso de ADN obtenido por un método convencional basado en la electroforesis en geles de agarosa seguida por la tinción con bromuro de etidio y su visualización con luz ultravioleta. Se encontró que la micropurificación de dos fragmentos de ADN, uno de 1,2 kpb que codifica el gen de resistencia a la kanamicina y el vector pUC19, mediante ambos métodos produjo el mismo —estadísticamente indistinguible ($P = 0,10$)—, número de colonias cuando éstos se utilizaron para transformar células competentes de *Escherichia coli* XL-1 Blue. Sin embargo, el número de transformantes obtenidos en ausencia del inserto fue significativamente menor cuando el vector de ADN fue micropurificado del gel de acrilamida mediante el método zinc-imidazol ($P = 0,0002$), lo que aumentó la probabilidad de encontrar colonias recombinantes en el experimento de clonación. Estos resultados estimularon el uso de la técnica basada en la tinción con zinc-imidazol para la micropurificación de ADN de alta calidad y para resolver las situaciones donde el ADN micropurificado convencionalmente de geles de agarosa (ej., genes de la proteína de membrana externa de clase 1 intacta o modificada en el lazo 5, y la proteína Opc de la cepa 866 de *Neisseria meningitidis),* falla en la generación de células recombinantes de *E. coli*.

Palabras claves: bromuro de etidio, clonación, electroforesis en gel, luz ultravioleta, tinción con zinc

*I*ntroduction

The most used method to recover individual DNA bands separated from agarose electrophoresis gel involves two steps: (i) gel observation under UV-light illumination after staining with ethidium bromide (EtBr) and (ii) DNA elution from the gel with the aid of established procedures [1]. Many cloning protocols based on this method warn of the dangers of UV-light exposure to the eyes, face, or skin and the impression emerges that photodamages of DNA is of minor importance [1]. However, in the last few years evidence has been accumulated that transillumination even during a brief period (1.5 min or less) heavily compromises the integrity and functionality of gel-separated DNA in a size-dependent manner as predicted by the target theory [2–6]. It appears therefore that this standard micropurification method is not completely satisfactory, although it is continually used, perhaps because of its simplicity and adequate sensitivity for many applications.

To avoid this drawback, we have recently developed a new method for the rapid micropurification of DNA from polyacrylamide gels. The method uses zinc and imidazole salts for the sensitive negative staining of DNA [6], followed by generation of gel microparticles to promote the rapid passive elution of DNA at room temperature [7]. As zinc-imidazole negative

1. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.

2. Cariello NF, Keohavong P, Sanderson BJS, Thilly WG. DNA damage produced by ethidium bromide staining and exposure to ultraviolet light. Nucleic Acids Res 1988;16:4157.

3. Hartman PS. Transillumination can profoundly reduce transformation frequencies. BioTechniques 1991;11:747–8.

✉ Corresponding author

staining does not require exposure of DNA to UV light, DNA recovered from stained gels is suitable for use in assays requiring high biological activity. We investigated here the suitability of our technique for the non-destructive micropurification of bioactive DNA, providing better data with respect to the transformation of *Escherichia coli* with DNA recovered from transilluminated ethidium bromide-stained gels.

Materials and Methods

Bacterial strains and growth conditions

E. coli XL-1 blue [*recA endA1 gyrA96 thi hsdR17* (r_k^- m_k^+) *supE44 relA1* λ^- *lac* (F´*pro AB lac* I^q Z*DM15* Tn*10*(tet)] [8] competent cells were used for the transformations of recombinants. They were cultured in Luria-Bertani (LB) medium [1], supplemented with ampicillin (50 µg/mL), when required, overnight at 37 ºC.

DNA procedures

Plasmid small-scale purification was carried out by the alkaline extraction method [1]. Routine recombinant DNA tecnology was performed as described [1]. Briefly, two plasmids, pUC19 (2.7 kbp) and pUC4K (4 kbp), were independently digested with *Bam*HI restriction enzyme to generate a linear pUC19 vector and a DNA fragment of 1.2 kbp (termed insert) corresponding to the kanamycine resistant gene. The digestion samples were separated on independent parallel lanes of a 1% low-gelling-temperature (LGT) agarose electrophoresis gel and then precisely localized by transillumination with 312-nm UV light for 1.5 min (or less) after staining the gel with ethidium bromide as usual [1]. Electrophoresis was carried out at 70 V constant voltage in the standard Tris-acetate-EDTA buffer, and was stopped when the bromophenol blue reached the gel bottom. In-solution DNA recovery was performed with the phenol-chloroform method [1] followed by ethanol precipitation. Next, the linear pUC19 plasmid was dephosphorylated by treatment with alkaline phosphatase [1] to avoid possible transformation with vector DNA molecules without insert (false positives) obtained in the ligation reaction, which would undoubtedly increase the probability of finding a colony with the desired insert (true positive). Finally, the gel-purified DNA fragments were left under standard ligation conditions [1] and 5 or 10 ng of the ligated DNA were used to transform *E. coli* XL-1 Blue competent cells. Alternatively, the same experiment was repeated except that the DNA was electrophoresed on a 5% polyacrylamide gel [1], negatively stained with zinc-imidazole [6] and subsequently recovered by rapid passive elution from gel microparticles [7] followed by ethanol precipitation [1].

Cloning experiments with different vectors and inserts

For cloning experiments the following four plasmids were employed: pM-80 [9], pM-82 [10], pM-107 [11] and pBlueScript II KS+ (Stratagene, USA). pM-80 and pM-82 plasmids were digested with *Xba*I–*Bam*HI and used as background for the cloning of different variants of the *porA* gene of *Neisseria meningitidis*, which were digested with the same restriction enzymes. pM-107 plasmid was digested with *Xba*I–*Bam*HI and used to clone the *opc* gene of *N. meningitidis*, also digested with the same restriction enzymes. pBlueScript II KS+ plasmid was digested with *Eco*RV and used to clone the streptokinase gene.

After the electrophoresis on a 5% polyacrylamide gel [1] both, vectors and inserts, were purified by the zinc-imidazole–based method [7] and ligated under standard ligation conditions [1]. The ligated DNA was used to transform *E. coli* XL-1 Blue competent cells.

Statistical analysis

The existence of a statistically significant effect of the staining method, DNA quantity, and vector:insert ratio on the results of the cloning experiment was determined by multi-factor analysis of variance (MANOVA) using the Statistica for Windows Release 4.2 package (StatSoft, Inc., USA).

Results and Discussion

Both DNA purification methods produced the same, statistically undistinguishable ($P = 0.10$) number of transformants (Tables 1 and 2) regardless of the separation matrix type (agarose, polyacrylamide) and on-gel DNA visualization method (EtBr + UV transillumination or zinc-imidazole). This result would in part be influenced by such manifold variability-associated parameters involved throughout the cloning experiment, as the use of different enzyme and competent cell batches.

4. Hardy E, Sosa A, Pupo E, Casalvilla R, Fernández-Patrón C. Zinc-imidazole positive: a new method for DNA detection after electrophoresis on agarose gels not interfering with DNA biological integrity. Electrophoresis 1996;17:26–9.

5. Gründeman D, Schömig E. Protection of DNA during preparative agarose gel electrophoresis against damage induced by ultraviolet light. BioTechniques 1996; 21:898–903.

6. Hardy E, Pupo E, Casalvilla R, Sosa AE, Trujillo LE, López E, *et al.* Negative staining with zinc-imidazole of gel electrophoresis-separated nucleic acids. Electrophoresis 1996;17:1537–41.

7. Castellanos-Serra L, Hardy E, Sánchez JC. Fast passive elution of DNA from zinc-imidazole negatively stained polyacrylamide gels. Anal Biochem 1998;257: 227–8.

8. Bullock WO, Fernández JM, Stuart JM. XL-1 Blue, a high efficiency plasmid transforming *Escherichia coli* strain with β-galactosidase selection. BioTechniques 1987;5:376–9.

Table 1. Results of cloning experiments with DNA recovered from agarose gels stained with ethidium bromide and visualized with UV light.

Quantity of ligated DNA used to transform cells	5 ng		10 ng	
Vector:insert ratio in the DNA ligation reaction	1:3	1:7	1:3	1:7
I	532 (1.06, 0.81)	318 (0.63, 0.48)	564 (0.56, 0.45)	774 (0.77, 0.62)
II	334 (0.66, 0.61)	294 (0.58, 0.53)	356 (0.35, 0.37)	570 (0.57, 0.59)
III	275 (0.55, 0.51)	482 (0.96, 0.90)	718 (0.71, 0.51)	756 (0.75, 0.54)
Range	275–774 (0.35–1.06;0.37–0.90)			
Grand Ave	498 (0.68, 0.58)			

Values are the number of transformants followed by the transformation frequency x10^{-2} and the stimulation factor in parenthesis, obtained for three independent experiments (I, II, III). The range of all values for the same DNA purification method (Range), and the average of these values (Grand Ave) are also shown. The stimulation factor was calculated as the ratio of the number of transformants contained on LB-ampicillin plates, which were obtained when the vector was ligated in the presence of the insert, relative to that in the absence of the insert (Table 3). Vector:plasmid pUC19 linearized by digestion with *Bam*HI. Insert: DNA fragment of 1.2 kbp encoding the kanamycine resistant gene, generated by digestion of the plasmid pUC4K with *Bam*HI. After micropurification from agarose stained with ethidium bromide, DNA fragments were dephosphorylated, ligated and used to transform *E. coli* XL-1 Blue competent cells.

Table 2. Results of cloning experiments with DNA recovered from polyacrylamide gels stained with the zinc-imidazole method.

Quantity of ligated DNA used to transform cells	5 ng		10 ng	
Vector:insert ratio in the DNA ligation reaction	1:3	1:7	1:3	1:7
I	258 (0.51, 32.25)	265 (0.53, 33.13)	406 (0.40, 18.45)	404 (0.40, 18.36)
II	550 (1.10, 1.69)	708 (1.42, 2.17)	1476 (1.48, 2.94)	1208 (1.21, 2.41)
III	348 (0.69, 2.13)	1000 (2.00, 6.13)	674 (0.67, 2.92)	2324 (2.32, 10.06)
Range	258–2324 (0.40–2.32, 1.69–33.13)			
Grand Ave	802 (1.06, 11.05)			

Values are the number of transformants followed by the transformation frequency x10^{-2} and the stimulation factor in parenthesis, obtained for three independent experiments (I, II, III). The range of all values for the same DNA purification method (Range), and the average of these values (Grand Ave) are also shown. Values were obtained as described in Table 1 except that DNA fragments were recovered from polyacrylamide gels stained by zinc-imidazole method.

It is worth noting, however, that the number of transformants obtained in the absence of the insert (Table 3) was significantly lower when the DNA vector was purified by the zinc-imidazole–polyacrylamide method ($P = 0.0002$), which would increase the probability of finding desired recombinant colonies in the cloning experiment. Some relevant factors influencing the different steps, which might explain these differences, are described below.

First, the number of colonies selected after transformation of cells with the *Bam*HI-digested DNA vector—linear DNA is not efficient in its ability to transform competent *E. coli* cells—was significantly lower ($P = 0.004$) when the zinc-imidazole–based purification method was used (Table 4, first row).

Second, the number of transformants obtained after cell transformation with the DNA vector digested with *Bam*HI and purified by gel electrophoresis, in the presence (or not) of T4 DNA ligase was significantly higher ($P = 0.018$) when the zinc-imidazole–polyacrylamide method was used (Table 4, second row). Although not verified in this work, some known causes for a lower efficiency of the T4 ligase enzyme to ligate the digested DNA vector purified from EtBr-UV–treated agarose as the following should not be discarded: (i) fragmentation or modification of the DNA vector induced by UV light, which could hinder recognition of the T4 ligation site and (ii) co-elution of impurities from the agarose gel that are able to inhibit T4 ligase activity.

These results have encouraged the use of our zinc-imidazole–based technique for the high quality purification of DNA for subsequent cloning experiments (Table 5), especially in situations where DNA purified from conventional LGT agarose may fail to induce transformation of *E. coli* cells. It is to be described that competent cells transformed with the resulting plasmids were capable of growing in media containing kanamycine, indicating the functionality of the protein that confers the resistance to the antibiotic (data not shown). Each new construct described in Table 5 was verified by either restriction analysis with appropriate enzymes or sequencing through both sides of the ligation junction (data not shown). In particular, expression of all the porin-based *N. meningitidis* mutants in *E. coli* W3110 accounted for more than 20% of the total cellular proteins (data not shown), as checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using monoclonal antibodies against class 1 subtypes P1.16 [12] and P1.19 [13]. The examined examples include a wide range of DNA sequences, suggesting that this technique should be applicable to DNA from other sources.

In summary, it was demonstrated that zinc-imidazole negative staining of DNA fragments separated from polyacrylamide gels followed by enhanced elution from gel microparticles can be used as a suitable alternative for the recovery of DNA in situations requiring maximum retention of DNA biological properties.

Table 3. Analysis of transformants obtained in cloning experiments performed in the absence of the insert.

Experiment number	A: DNA from agarose stained with ethidium bromide		B: DNA from gel stained by the zinc-imidazole method		Ratio A:B	
	5 ng	10 ng	5 ng	10 ng	5 ng	10 ng
I	656 (1.31)	1246 (1.25)	8 (0.01)	22 (0.02)	82.00	56.64
II	552 (1.10)	962 (0.96)	326 (0.65)	502 (0.50)	1.69	1.92
III	534 (1.07)	1398 (1.40)	163 (0.32)	231 (0.23)	3.28	6.05
Range	552–1398 (0.96–1.40)		8–502 (0.01–0.65)		1.69–82	
Grand Ave	891 (1.18)		209 (0.29)		25	

Values of A and B are the number of transformants followed by the transformation frequency $x10^{-2}$, obtained for three independent experiments (I, II, III). The range of all values for the same DNA purification method (Range), and the average of these values (Grand Ave) are also shown. Vector:plasmid pUC19 linearized by digestion with *Bam*HI. After micropurification from agarose stained with ethidium bromide or from polyacrylamide gels stained with zinc-imidazole, the DNA vector recovered was dephosphorylated and ligated. An amount of 5 ng or 10 ng of the ligated DNA was used to transform *E. coli* XL-1 Blue competent cells.

Table 4. Statistical analysis of factors affecting the different steps of the cloning experiment.

Factor	Polyacrylamide gel stained by zinc-imidazole method	Agarose gel stained with ethidium bromide	Probability for equal means
Contaminating native DNA vector	342[a]	1009[a]	0.004
T4 DNA ligase activity	12.8[b]	6.1[b]	0.018

[a]Values are the number of transformants obtained after transformation of cells with 5–10 ng *Bam*HI-digested DNA vector purified by gel electrophoresis. Values represent the average of six independent experiments.

[b]Values are the ratio of the number of transformants obtained after transformation of cells with *Bam*HI-digested DNA vector purified by gel electrophoresis and ligated with T4 ligase, relative to that obtained without T4 ligase treatment. Values represent the average of six independent experiments.

Table 5. Cloning experiments with DNA recovered from zinc-imidazole stained gels[a].

Vector	Size (kbp)	Insert	Size (kbp)	Stimulation factor[b]	Transformation frequency ($x10^{-2}$)
pM-82	4.2	*porA*(P1.16)	1.1	18	4.4
		porA(P1.19,15)	1.15	12	6.0
		porA(P1.16Δ5)	1	12	0.4
pM-107	4.75	*opc*	0.75	28	1.4
pM-80	3.9	*porA*(P1.16)	1.1	3	0.1
		porA(P1.19,15)	1.1	40	3.0
pBlueScript^{R} II KS(+)	2.96	*sk*	1.3	25	3.2

[a]Vectors pM-80, pM-82, pM-107 and pBlueScript II KS+ and all of the inserts were purified by the zinc-imidazole–based method [7]. Values represent the average of two independent experiments. *porA*(P1.16), *porA*(P1.19,15), *porA*(P1.16Δ5) and *opc* are the genes for: the class 1 outer membrane proteins from the 866 strain; from the B385 strain; from the 866 strain with a deletion in loop 5 and Opc-protein from *N. meningitidis,* respectively. *sk:* Gene for recombinant streptokinase.

[b]The stimulation factor was calculated as the ratio of the number of transformants contained on LB-ampicillin plates, which were obtained when the vector was ligated in the presence of the insert, relative to that in the absence of the insert.

Acknowledgements

We express our greatest recognition to anonymous reviewers for valuable suggestions which improved this presentation.

9. Carmenate T, Álvarez A, González S, Musacchio A, Niebla O, Delgado M, *et al.* Cloning and expression of *opc,* the gene encoding the outer membrane protein 5c from *Neisseria meningitidis.* Purification and immunological characterization of the recombinant polypeptide. In: Evans, Yost, Maiden and Feavers, editors. Proceedings of the Ninth International Pathogenic Neisseria Conference; 1994 Sep 26–30; Winchester, England; 1994. p.101–2.

10. Niebla O, Álvarez A, González S, Musacchio A, Delgado M, Silva R, *et al.* Cloning and expression of *porA,* the gene encoding the class 1 outer membrane protein from *Neisseria meningitidis.* Purification and immunological characterization of the recombinant polypeptide. In: Evans, Yost, Maiden and Feavers, editors. Proceedings of the Ninth International Pathogenic Neisseria Conference; 1994 Sep 26–30; Winchester, England; 1994. p.85–6.

11. Carmenate T, Delgado M, Pérez M, Guillén G. Recombinant Opc reconstituted into liposomes elicits opsonic antibodies. In: Zollinger, Frasch and Deal, editors. Proceedings of the Tenth International Pathogenic Neisseria Conference; 1996 Sep 8–13; Baltimore, USA; 1996. p.143–4.

12. McGuinness BT, Clarke IN, Lambden PR, Barlow AK, Poolman JT, Jones DM, *et al.* Point mutation in meningococcal *porA* gene associated with increased endemic disease. Lancet 1991;337:514–7.

13. Cruz S, Musacchio A, Fernández de Cossío ME, Ohlin M, Nazábal C, Freyre M, *et al.* Mouse monoclonal antibodies against outer membrane proteins of the vaccine strain of *Neisseria meningitidis* B:4:P1.15. Minerva Biotechnology 1998;10:65–70.

Received in December, 1999. Accepted for publication in September, 2000.