Ligation-independent cloning of PCR products (LIC-PCR)

Charalampos Aslanidis and Pieter J.de Jong*
Lawrence Livermore National Laboratory, Biomedical Sciences Division L-452, PO Box 5507, Livermore, CA 94550, USA

Received June 12, 1990; Revised and Accepted September 20, 1990

ABSTRACT

A new procedure has been developed for the efficient cloning of complex PCR mixtures, resulting in libraries exclusively consisting of recombinant clones. Recombinants are generated between PCR products and a PCR-amplified plasmid vector. The procedure does not require the use of restriction enzymes, T4 DNA ligase or alkaline phosphatase. The 5'-ends of the primers used to generate the cloneable PCR fragments contain an additional 12 nucleotide (nt) sequence lacking dCMP. As a result, the amplification products include 12-nt sequences lacking dGMP at their 3'-ends. The 3'-terminal sequence can be removed by the action of the (3'→5') exonuclease activity of T4 DNA polymerase in the presence of dGTP, leading to fragments with 5'-extending single-stranded (ss) tails of a defined sequence and length. Similarly, the entire plasmid vector is amplified with primers homologous to sequences in the multiple cloning site. The vector oligos have additional 12-nt tails complementary to the tails used for fragment amplification, permitting the creation of ss-ends with T4 DNA polymerase in the presence of dCTP. Circularization can occur between vector molecules and PCR fragments as mediated by the 12-nt cohesive ends, but not in mixtures lacking insert fragments. The resulting circular recombinant molecules do not require in vitro ligation for efficient bacterial transformation. We have applied the procedure for the cloning of inter-ALU fragments from hybrid cell-lines and human cosmid clones.

INTRODUCTION

For many applications, the polymerase chain reaction (1) has replaced molecular cloning as the method of choice for the rapid amplification and isolation of specific DNA sequences from genomic DNA. If the PCR results in a single product or if the desired product can readily be separated from contaminating products, there is often no need for cloning. By contrast, complex PCR product mixtures still require cloning for the isolation of specific products, e.g. for use as probes. This is for instance the case for PCR libraries established from micro-dissected chromosomes (2, 3) and for inter-ALU PCR (4, 5). Cloning can be performed by conventional procedures such as the use of restriction sites present in the PCR product or by blunt end cloning of the PCR products. However, the cloning of PCR products does often prove to be less straightforward than anticipated. Blunt end cloning is rendered more difficult by the addition of a nucleotide to the 3' ends of the PCR product by Taq polymerase (6). Removal of this 3' nucleotide, e.g. using the Klenow fragment of DNA polymerase I, is a prerequisite for the blunt-end cloning of the PCR product (7). Also, it is necessary to prevent high non-recombinant backgrounds by treating the vector with alkaline phosphatase. Even with these preventions, a sizeable fraction of the clones will lack inserts. This may not present a major problem if a convenient screening procedure is available, such as the functionality of the lacZα gene segment in pUC plasmids (8). But these screens provide only a crude distinction. White clones resulting from lacZα inactivation are often caused by frame-shift mutations in the vector inadvertently introduced during the cloning process. As an alternative to blunt end cloning, restriction sites can be added to the 5' tails of the PCR primers, thus permitting cohesive end cloning. Our own results as well as anecdotal reports from other scientists indicate that this procedure is often inefficient.

Recently, procedures for the amplification of sequences flanked by ALU-repeats have been developed (4, 5, 9,). For this purpose human DNA from hybrid cell lines or cosmids were amplified using a single ALU-repeat primer. A single ALU primer permits PCR amplification of the human DNA provided two flanking ALU repeats (10, 11, 12) are present within close range (up to 3 Kbp) in a palindromic orientation. ALU-PCR fragment sizes generally range from 150−3000 bp. For the best ALU-primers, an average of about one distinct PCR product per 50 Kbp of genomic sequence can be generated under conditions not generating products from the Chinese hamster genome. These ALU-PCR products represent sequences present in the human segment(s) from the somatic cell hybrids. Although we could establish libraries in plasmid vectors using blunt end cloning of PCR products or sticky end cloning by the use of a restriction site at the 5' end of the ALU-primer, the cloning efficiency was often less than desirable. Therefore, we have developed a more effective method for the cloning of these products, that does not involve restriction enzymes or DNA ligase. In essence, long single-stranded tails are created at the ends of the ALU-PCR products and the linear plasmid vector. The ss-ends present at the PCR fragments are complementary to those attached to the

* To whom correspondence should be addressed

vector, thus permitting non-covalent bi-molecular associations e.g. circularization between two molecules. The vector has identical, non-complementary tails at either end, preventing the formation of circular forms consisting of vector only. The bi-molecular ('recombinant') forms are used to transform E.coli; single-molecule ('non-recombinant') transformants are strongly reduced due to the poor transformation efficiency of linear plasmid DNA in E.coli. Transformation efficiencies for linear plasmid are 10^2 to 10^3 fold reduced in rec^+ bacteria and $10^3-4\times10^4$ fold reduced in rec^- bacteria (13).

Using the ligation-independent cloning (LIC) method, we have now established complex PCR product libraries.

MATERIALS AND METHODS

Enzymes and reagents

Taq polymerase was from Perkin Elmer-Cetus; T4 DNA polymerase and dNTPs were from Boehringer Mannheim. Competent bacteria ('Max Efficiency' DH5α, DH5αF'IQ or DH10B) and the 123-bp ladder were purchased from BRL. Oligonucleotides were synthesized using an ABI-380B DNA synthesizer and purified by HPLC. The following primers were synthesized: Alu-primer PDJ83 (5'-GATGGTAGTAGGCCAC-TGCACTCCAGCC) and vector primers PDJ80 (5'-CCTACTA-CCATCGGATCCCCGGGT) and PDJ81 (5'-CCT-ACTACC-ATCGTCGACCTGCAG).

Amplification of vector and human DNA

All PCR reactions were performed in 50 μl mixtures covered with mineral oil using the following buffer conditions: 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. Amplification was for 35 cycles on a Perkin Elmer Cetus thermal cycler as follows: 1 min. 93°C, 1 min. 60°C and 1 min. at 72°C. The vector was amplified starting with 1 ng of circular or XbaI-linearized pUC119 plasmid (14) and 1 μg of each PCR primer (PDJ80, PDJ81). After an initial boiling of 5 min, 2 units of Taq polymerase were added and PCR was started. An additional 5 min. at 72°C was used for the last cycle. Inter-ALU sequences were amplified using 1 μg of ALU primer (PDJ83), 10 ng of genomic DNA from a Chinese hamster/Human hybrid cell line (UV5HL9−5B) or from Chinese hamster cells(UV5, 15) and 1 unit of Taq polymerase. The hybrid line is a subclone from UV5HL9 (15) with essentially the same properties: a single human chromosome 19 in a hamster background. The same cycling program was used for the inter-ALU PCR as described for the vector PCR, except that no initial boiling was used. Products from PCR reactions were usually analyzed by electrophoresis in 2 to 3% agarose gels (NuSieve GTG) in TAE buffer with ethidium bromide (8).

T4 DNA polymerase treatment

Vector and inter-ALU PCR products were purified using separate procedures prior to the exonuclease digestion. The amplified vector was purified by electrophoresis in TAE buffer. The vector fragment was then recovered from the agarose gel using a glass powder suspension ('Gene-CleanII' from BIO101, San Diego) as recommended by the supplier. ALU-PCR fragments were purified using 'Gene-Clean' without prior electrophoresis.

For the generation of the single stranded tails, the purified DNA preparations were treated with 2 units T4 DNA polymerase in a 200 μl volume (33 mM Tris-acetate pH 8.0, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 μg/ml bovine serum albumin) in the presence of dGTP (0.5 mM) for the ALU-PCR products or dCTP (0.5 mM) for the vector. After incubation for 20 min at 37°C the mixtures were heated for 10 min at 65°C and DNA was purified using 'Gene-Clean'. The vector was diluted to 3 ng/μl and the ALU-PCR products to 6 ng/μl.

Cloning and transformation

A 3μl aliquot of T4 DNA polymerase treated pUC119 (9 ng) was combined with 4 μl of T4 DNA polymerase treated ALU-PCR products (24 ng) in a 20 μl volume (25 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM ATP). After an one hour incubation at room temperature, 5μl were used to transform 50 μl competent cells (16) using BRL-recommended procedures. SOC medium (0.45 ml) was added and 100 μl of the bacterial suspension was plated on LB plates (supplemented with 100 μg/ml of ampicillin, 0.004% XGal and 100 μM IPTG) after 1 hour at 37°C.

PCR of transformants

Transformants were checked for the presence of recombinant plasmids by amplification using the ALU-PCR primer. Bacterial colonies were transferred into the PCR mixture using disposable pipette tips. Amplification was for 35 cycles of the standard cycling program and 5μl aliquots were analyzed by agarose gel electrophoresis.

RESULTS

Outline of the procedure

Initial attempts to clone ALU-PCR products using either blunt-end or cohesive end ligations resulted in a low cloning efficiency and a high non-recombinant background in the product libraries (data not shown). To improve the cloning efficiency and reduce the background, we designed a new procedure based on the use of 12-nt long cohesive ends at the cloneable PCR products as well as the vector. This involves the amplification of the entire plasmid vector pUC119 with primers complementary to regions of the multicloning site. These two primers (PDJ80, PDJ81) have 12 identical nucleotides at their 5'-ends, lacking dGMP residues, that do not hybridize to the vector (Fig.1). PCR products generated with these primers thus lack dCMP residues at their 3'-ends. After amplification, the PCR product is treated with the (3'→5') exonuclease activity of T4 DNA polymerase in the presence of dCTP to degrade the DNA from the 3'-ends up to the first dCMP residue.

ALU-PCR products are generated using a primer (PDJ83), which has its 17 3' nucleotides in common with the human ALU repeat sequence (positions 240−256 of the consensus ALU repeat sequence (12). The 5'-end of the ALU-primer is complementary to the 5'-ends of the vector primers(PDJ80 and 81) and thus lacks dCMP in the 5' terminal 12 nt-sequence. Consequently, single stranded tails can be generated with T4 DNA polymerase in the presence of dGTP.

Cloning of ALU-PCR products

T4 DNA polymerase treated vector and ALU-PCR products can be mixed and are expected to generate non-covalently linked hybrid products by virtue of the 12 nucleotide complementary tails. If these hypothetical hybrid molecules stay associated during bacterial transformation and are repaired upon entry into the recipient cell, recombinant clones might be established.

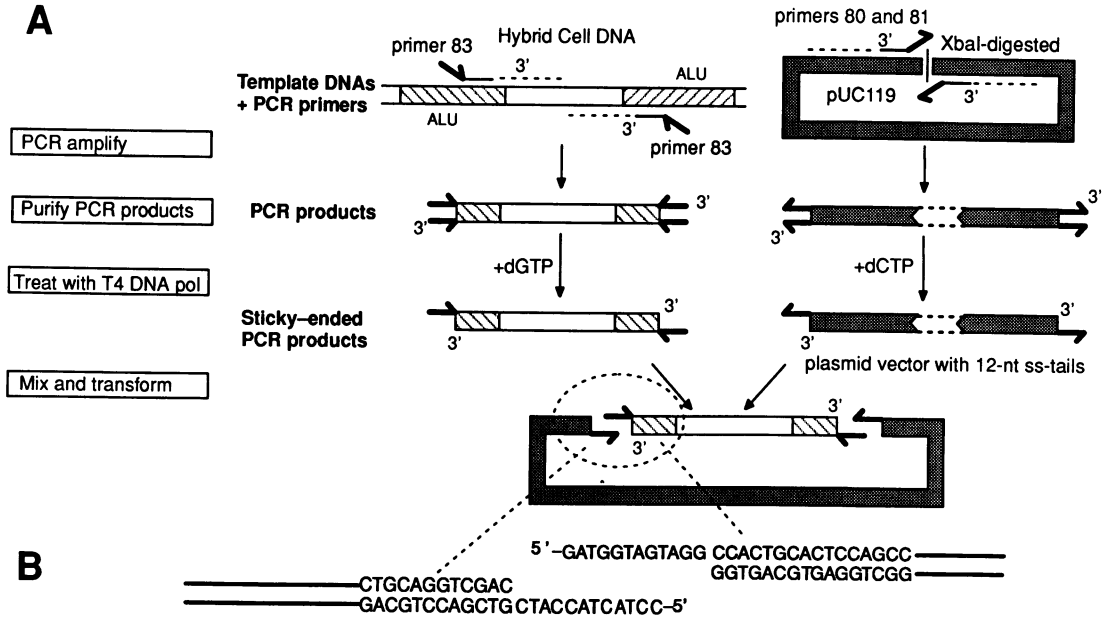

Fig. 1. Generation of defined single stranded ends on PCR products. Inter-ALU sequences are amplified from the human genome using primer PDJ83 partly homologous to the consensus ALU sequence (12). Vector pUC119 is amplified with primers PDJ80 and 81 homologous at the 3'-end to the multiple-cloning sequence. The PCR products are digested with the (3'→5') exonuclease associated withT4 DNA polymerase in the presence of dGTP (inter-ALU's) or dCTP (pUC119) to create 12-nt single-stranded ends. The 5'-overhanging ends from vector and ALU-PCR products are complementary (panel B) and allow dimeric circles to be formed without requiring DNA ligase.

To test this cloning procedure, DNA from a human/Chinese hamster hybrid cell line (UV5HL9−5B) was used to generate cloneable ALU-PCR fragments. This hybrid line contains a single human chromosome 19 in addition to the rodent chromosomes (15). Amplification of hybrid genomic DNA with ALU primer PDJ83 results in a complex mixture of PCR fragments, as is evident upon electrophoretic analysis in agarose gels (Fig. 2 , lane 3). The size of the DNA fragments is in the range of 150−3000 bp. No PCR amplification was observed when Chinese hamster DNA was used as template (Fig.2, lane 2). Circular vector DNA (pUC119) was used as template for vector amplification.

The ALU-PCR products and the linear vector PCR products were treated with the T4 DNA polymerase to generate the complementary single-stranded tails. Vector (pUC119, 9 ng) and inter-ALU fragments (24 ng) were mixed in a 20 μl volume and incubated for one hour to allow the formation of hybrid products, e.g. circles containing single insert and vector molecules. The mixture was directly used for bacterial transformation without first ligating the products. A fraction of the transformed cells was plated on LB plates supplemented with ampicillin, XGal and IPTG. This resulted in 140 white and 110 blue transformants (Table 1). Control transformations using only amplified vector (with or without T4 DNA polymerase treatment) result in approximately 70 blue and no white transformants. This indicates that minute amounts of circular plasmid have survived the PCR procedure and have been co-purified during the agarose electrophoresis. Additional control experiments where either the vector or the ALU-PCR products, or both, were not treated with T4 DNA polymerase result in similar amounts of blue colonies (range of 57−96) but exhibit a considerably lower number of white transformants (range of 1−5).

Only if both vector and ALU-PCR fragments had been treated

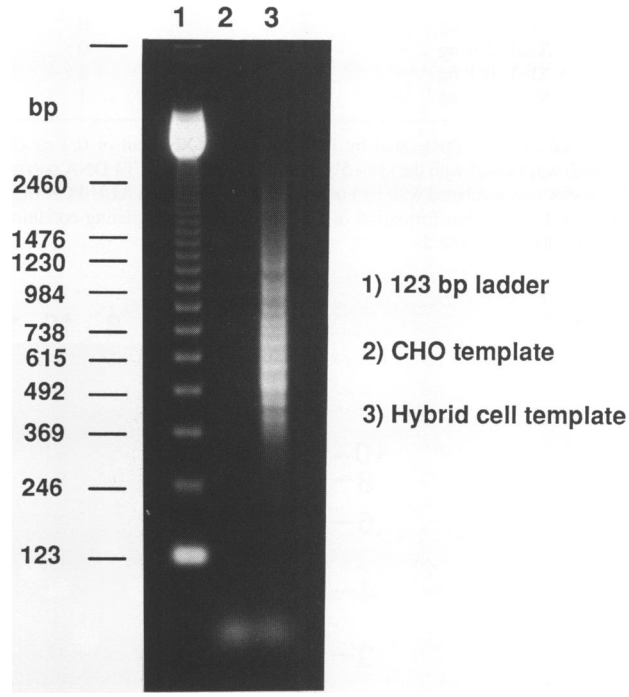

Fig. 2. ALU-PCR from hybrid cell DNA. PCR products were generated with ALU primer PDJ83 from Chinese hamster DNA (lane 2) or hybrid cell DNA (lane 3) and were analyzed by gelelectrophoresis in 1.4% agarose. Lane 1 contains the 123-bp ladder.

with the exonuclease associated with T4 DNA polymerase, a high number of transformants (white colonies) was obtained, indicating the involvement of 12 nucleotide cohesive ends (Table 1). The blue colonies in the control experiments are likely the result of

residual amounts of uncut pUC119 vector used as template for the amplification, even though the amplified form was purified from the gel.

To eliminate the non-recombinant background, vector DNA (1 ng) was linearized with XbaI (Fig. 1) or a lower quantity (0.1 ng) of circular plasmid was used as template for PCR amplification. The linearized vector was a better PCR substrate, resulting in larger quantities of product (data not shown). Lowering the DNA amount of pUC119 in the PCR by 100 fold (10 ng versus 0.1 ng, Tables 1 and 2) lowered the blue transformants dramatically (25 fold) but did not eliminate them (see also Table 1). By contrast, linearization of the vector prior to PCR eliminates blue transformants (Table 2). Although no blue transformants appeared on the plates using XbaI-cut pUC119 vector in combination with ALU-PCR products, some faint blue colonies were observed. To characterize the transformants, 17 white and 10 faint blue colonies were tested for the presence of ALU-PCR inserts. The clones were analyzed by agarose gelelectrophoresis following PCR amplification with the primer PDJ83. In all 27 transformants, amplification of a distinct fragment occurred (Fig.3). The faint blue transformants appeared to contain recombinant plasmids with smaller inserts than the white transformants, suggesting that the inserts possibly contain an open reading frame allowing translational read-through of the lacZα fragment. Blue transformants from the initial experiment (Table 1) were also amplified and show in all cases a low-intensity 750 bp PCR product (data not shown) which could not be detected in the case of white transformants. Further experiments indicate that this fragment is generated from the vector and not from the E.coli chromosomal DNA: PCR amplification of DH5α does not generate this fragment (Fig.3). Further characterization of the putative recombinants by restriction analysis of plasmid DNA indicates that all plasmids have an insert, which can be released by restriction enzymes cutting in the multiple cloning site (data not shown).

We have used 21 ALU-PCR clones to determine their human or hamster origin (data not shown). They have been hybridized individually to Southern blots containing ALU-PCR products from UV5HL9−5B (hybrid) and UV5 (hamster), generated with the same PCR primer. Seventeen clones appeared to be human with the remaining four being of hamster origin, indicating that the PCR process very preferentially amplifies human sequences. The human clones were also used to probe an arrayed portion

Table 1. Ligation-independent cloning.

	Vector	ALU-PCR product	White Colonies	Blue Colonies
1)	no T4	n.a.	0	72
2)	no T4	no T4	2	82
3)	no T4	T4	1	57
4)	T4	n.a.	0	70
5)	T4	no T4	5	96
6)	T4	T4	140	110

The number of white and blue transformants is given for six different experiments. Vector (9ng) and ALU-PCR fragments (24ng) were combined in a 20μl volume at room temperature; after a one hour incubation, 5 μl was used for transformation of DH10B, and 20% of the transformed cell suspension was plated on LB ampicillin plates. Linear pUC119 DNA was obtained by PCR starting with 10ng circular plasmid. Abbreviations are: T4, T4 DNA polymerase treatment; no T4, no T4 DNA polymerase treatment; n.a., no DNA added.

Table 2. Elimination of background transformants.

	Vector	ALU-PCR product	White Colonies	Blue Colonies
1)	+XbaI; 1.0 ng	−	1	0
2)	+XbaI; 1.0 ng	+	36	0
3)	−XbaI; 0.1 ng	−	0	3
4)	−XbaI; 0.1 ng	+	57	4

Amplified pUC119 (generated by PCR from 1 ng XbaI-cut or 0.1 ng circular plasmid) was treated with the (3′→5′) exonuclease activity of T4 DNA polymerase. The vector was incubated with (+) or without (−) T4-treated ALU-PCR fragments and then used for transformation of DH10B. Mixing and plating conditions are as described for Table 1.

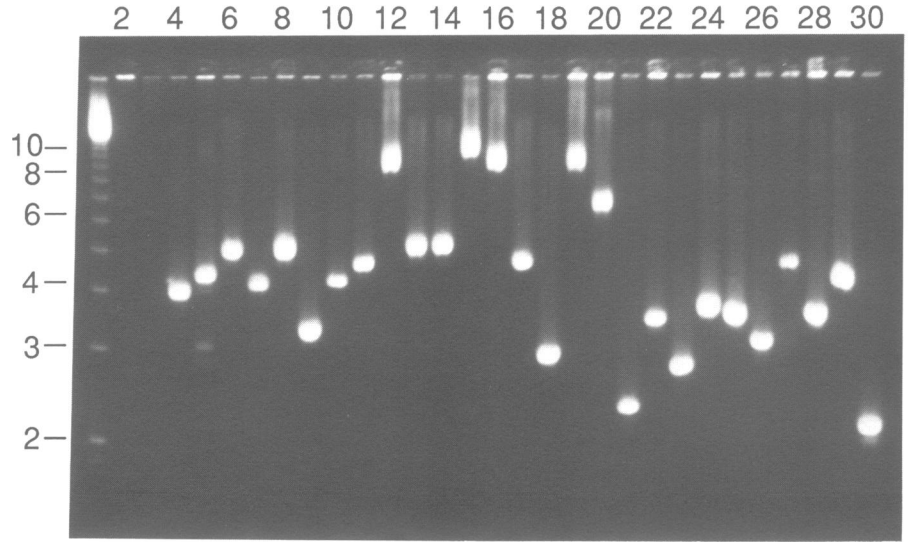

Fig. 3. Characterization of clones by agarose gelelectrophoresis. ALU-PCR products from 17 white (lanes 4−20) and 10 faint blue (lanes 21−30) transformants using the ALU primer PDJ83 are separated on a 3% agarose gel (NuSieve-GTG). Lane 2 contains the products from the DH5α controlamplification, lane 3 contains the DH5α pUC119 controlamplification. Lane 1 is the 123 bp ladder from BRL. Amplification was performed using bacterial material in the PCR mixture (see Materials and Methods).

of the same library (384 clones) to determine the redundancy. The results indicate an average of 0.7 cross-hybridizing colonies per probe, indicating a high complexity of the library.

The new cloning procedure has successfully been applied for a number of further cloning experiments. Transformed cells were no longer screened for lacZα function. As of now, 120 non-screened colonies have been analyzed and were in all cases found to contain inter-ALU inserts.

Since PCR amplification is known to be mutagenic, the function of a non-selected genetic element, the M13 replication origin, was checked. To this end, single stranded DNA was prepared from 12 transformants of a F′ host (DH5αF′IQ). Upon super-infection with phage M13-KO7 (14), single stranded DNA could be isolated for all plasmids tested. Also, the DNA could be sequenced using the fluorescently-tagged universal M13 primer (17), indicating that the primer binding site was sufficiently intact. In a total of 9 out of 10 sequenced cases the expected vector/insert junctions have been demonstrated. In one case, the ALU primer sequence was directly repeated in tandem followed by insert sequence (data not shown).

DISCUSSION

We have described a new method to clone PCR fragments which does not require the use of restriction enzymes and DNA ligase. The method relies instead on the (3′→5′) exonuclease associated with T4 DNA polymerase to generate long cohesive ends on both the vector and the cloneable DNA fragments. The cohesive ends at either vector-end are non-complementary thus reducing circularization of vector molecules. Nevertheless, when such plasmid molecules are treated with ligase and then used for transformation, a low number of 'background' recombinants are observed after screening for lacZα activity (data not shown). These recombinants lack insert DNA and likely have frame-shift mutations at the cloning site, e.g. resulting from the low-fidelity of T4 DNA ligase under some conditions (18, 19, 20) or the presence of low levels of contaminating nucleases. Similar observations are rather common for cloning in plasmid vectors using restriction enzymes and DNA ligase. The 12 nucleotide 5′-extending vector ends created by our procedure, permit the formation of relatively stable duplexes with cohesive tails from the PCR products, thus eliminating the need for a ligation step. It was expected that the fidelity of the cloning process would increase in the absence of a ligation step. Non-covalently joined recombinant molecules formed during the pre-incubation can only be established as clones if the molecular joints survive the transformation step and become covalently attached upon entry in the recipient cells. Double-hit transformation with two molecules and the subsequent joining of the ends might also be possible, but should be rare at the low DNA concentrations employed in our experiments. The stability of the correct bi-molecular interactions as compared to the considerably lower stability of non-complementary end-interactions is therefore expected to guard against background recombinants. This prediction appears to be true. Transformants containing 'background' recombinants (rearranged vector) have not been observed in our experiments. Furthermore, transformants resulting from non-recombinant vector can also be eliminated provided that the vector is linearized prior to PCR. The absence of a non-recombinant background reduces the need to screen for recombinants and thus simplifies the procedure. The new cloning procedure is rather simple since only a single type of enzymatic reaction is required prior to transformation and results in an acceptable cloning efficiency: about 2 to 5×10^5 recombinants/μg of vector.

Conventional cloning procedures such as blunt-end cloning require that the vector be dephosphorylated to prevent a high non-recombinant background. PCR does not provide sufficiently blunt-ended molecules, necessitating additional polymerase or exonuclease treatments to polish the ends of PCR products (6, 7). Cloning procedures employing restriction enzymes require recognition sites at a sufficient distance from the ends of the PCR product to permit efficient cutting (21). Dephosphorylation of the vector might also be required if directional cloning is impossible. Cohesive-end cloning of PCR products appears often inefficient for unknown reasons (unpublished observations and anecdotal reports from others). After finishing our experiments, two more ligase-free cloning procedures were published (22, 23), using overlaps between vector and insert created by denaturation and reannealing steps. While such reannealing steps may be efficient for the subcloning of defined sequences, a lower efficiency is expected for the reannealing of complex PCR mixtures for library construction. Furthermore, these procedures employ PCR primers containing additional 5′-tails of 24 nt (22) or 30 nt (23), as compared to only 12 nt in our method, adding to the cost of the experiment. Our LIC-PCR procedure appears to be at the very least a good and reliable alternative for the more conventional cloning procedures.

ACKNOWLEDGEMENTS

Chira Chen is gratefully acknowledged for providing the conditions for the ALU-PCR and for the preparation of excellent pictures. We thank Tony Carrano for critical reading of the manuscript and advice. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract No. W-7405-ENG-48. C.A was supported by a 'Gene Technology' fellowship from DAAD.

REFERENCES

1. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R. and Horn, G.T. (1988) *Science* **239**, 487–491.
2. Luedecke, H.J., Senger, G., Claussen, U. and Horsthemke, B. (1989) *Nature* **338**, 348–350.
3. Johnson, D.H. (1990) *Genomics* **6**, 243–251.
4. Brooks-Wilson, A.R., Goodfellow, P.N., Povey, S., Nevanlinna, H.A., de Jong, P.J. and Goodfellow, P.J. (1990) *Genomics* **7**, 614–620.
5. Cotter, F.E., Hampton, G.M., Nasipuri, S., Bodmer, W.F. and Young, B.D. (1990) *Genomics* **7**, 257–263.
6. Clark, J.M. (1988) *Nucleic Acids Res.* **16**, 9677–9686.
7. Hemsley, A., Arnheim, N., Toney, M.D., Cortopassi, G. and Galas, D.J. (1989) *Nucleic Acids Res.* **17**, 6545–6551.
8. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
9. Nelson, D.L., Ledbetter, S.A., Corbo, L., Victoria, M.F., Ramirez-Solis, R., Webster, T.D., Ledbetter, D.H. and Caskey, C.T. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6686–6690.
10. Jelinek, W.R. and Schmid, C.W. (1982) *Ann. Rev. Biochem.* **51**, 813–844.
11. Jurka, J. and Smith, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4775–4778
12. Kariya, Y., Kato, K., Hayashizaki, Y., Himeno, S., Tarui, S. and Matsubara, K. (1987) *Gene* **53**, 1–10.
13. Conley, L.C. and Saunders, J.R. (1984) *Mol. Gen. Genet.* **194**, 211–218.
14. Viera, J. and Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
15. Stallings, R.L., Olson, E., Strauss, W., Thompson, L.H., Bachinsky, L.L. and Siciliano, M.J. (1988) *Am. J. Hum. Genet.* **43**, 144–151.
16. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.

17. Connell, C., Fung, S., Heiner, C., Bridgham, J., Chakerian, V., Heron, E., Jones, B., Menchen, S., Mordan, W., Raff, M., Recknor, M., Smith, L., Springer, J., Woo, S. and Hunkapillar, M. (1987) *BioTechniques* **5**, 342–348.
18. Goffin, C., Bailly, V. and Verly, W.G. (1987) *Nucleic Acids Res.* **15**, 8755–8771.
19. Wiaderkiewicz, R. and Ruiz-Carrillo, A. (1987) *Nucleic Acids Res.* **15**, 7831–7848.
20. Wu, D.Y. and Wallace, R.B. (1989) *Gene* **76**, 245–254.
21. Scharf, S.J., Horn, G.T. and Erlich, H.A. (1986) *Science* **233**, 1076–1078.
22. Shuldiner, A.R., Scott, L.A. and Roth, J. (1990) *Nucleic Acids Res.* **18**, 1920.
23. Jones, D.H., Sakamoto, K., Vorce, R.L. and Howard, B.H. (1990) *Nature* **344**, 793–794.