Technical Tips Online, Vol. 2, 1997

Increased cloning efficiency by cycle restriction–ligation (CRL)

Carsten Pusch, Holger Schmitt and Nikolaus Blin

Institute of Anthropology and Human Genetics, Eberhardt-Karls-University, 72074 Tübingen, Germany

Keywords: Cloning & sequencing

▼Subcloning DNA fragments in plasmid vectors involves time-consuming steps including the preparation (e.g. linearization and dephosphorylation) of vector DNA. An additional problem is the selection of suitable ligation temperatures for cloning of blunt- or sticky-end fragments. One attractive innovation to circumvent these difficulties is temperature cycle ligation (TCL; Ref. 1). Briefly, the ligation reaction mix is simply cycled for several hours in a PCR machine between 10°C, which favours strand annealing, and 30°C, which favours enzymatic joining. The result is more ligated molecules and consequently an improved transformation efficiency as compared with a standard incubation at a single compromise temperature. We have been successfully using a comparable ligation program (Fig. 1) on a thermocycler (Biometra), but in addition, we applied three different temperature cycle steps. Cycling through the steps given in Fig. 1 offers a good balance between reaction conditions for DNA annealing and ligation. In order to reduce the steps of the conventional procedure to a single reaction, we established a new protocol. The addition of restriction enzymes to open self-ligated vector molecules results in highly efficient ligation of blunt- or sticky-end inserts of sizes ranging from 0.1 kb up to 7.0 kb into a wide range of plasmid vectors. Therefore we introduced three modifications in our established TCL program, depending on the restriction enzyme's working temperature and with respect to best ligation conditions for blunt- (Fig. 2a) and sticky-end reactions (Fig. 2b). Vectors of different sizes used to establish our CRL protocols were pUC19 (2.7 kb), pBluescript IISK- (2.9 kb), pBR322 (4.3 kb), and the expression vector pGal4 (4.6 kb).

Blunt-end DNA fragments were generated digesting cosmids (Ref. 2) with restriction endonucleases *Alu*I, *Eco*RV, *Hae*III, *Pvu*II, *Rsa*I (Biolabs, Boehringer Mannheim) or performing PCR using Pfu polymerase (Stratagene), which offers 3′ to 5′ proofreading exonuclease activity. Fragments were isolated from agarose or polyacrylamide gels and purified by the freeze–squeeze technique (Ref. 3) or the QIAquick kit (Qiagen). For setting up blunt-end cloning, we mixed 20 U *Sma*I, 1 U T4 DNA ligase (Eurogentech), 500–800 ng circular vector DNA, 100 ng glycogen per microlitre of reaction volume in appropriate CRL buffer (5 mM Tris-HCl, pH 7.7, 0.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM DTT, 50 μg/ml BSA, 0.1 mM ATP). All plasmid vectors were linearized during the reaction by the restriction enzyme *Sma*I (AGS). The DNA fragments to be cloned were added to give a ratio of 4:1 vector molecules to fragment molecules. The reaction was performed according to the program given (Fig. 2a).

Sticky-end DNA fragments were generated by *Tsp*509I, *Taq*I, *Sau*3AI and *Nla*III, purified as described above, and cloned into the corresponding 6-bp site (*Eco*RI, *Acc*I, *Bam*HI and *Sph*I) of appropriate vectors. The CRL reaction mixture contained 10 U of the suitable 6-bp cutter, 0.3 U T4 DNA ligase, 150–300 ng circular vector DNA, 50 ng glycogen per microlitre of reaction volume in sticky CRL buffer (3 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 10 mM KCl, 0.1 mg/ml BSA, 0.1 mM ATP) and each reaction was covered with 50 μl light mineral oil. Here, the best ratio of vector:fragment molecules was 2:1. The CRL protocol we used is shown (Fig. 2b).

The PCR program for asymmetric CRL cloning (e.g. fragments generated by two different restriction enzymes) was established using the restriction endonucleases *Sma*I and *Eco*RI in a cycle program randomly alternating between 15°C and 37°C. Each extreme temperature setting is applied for 3 min. Final steps to complete linearization of the vector molecules were added to the program for 2 h at the appropriate reaction temperature (25°C or 37°C, respectively) in order to minimize the background resulting from clones displaying vector self-ligation or dimerization.

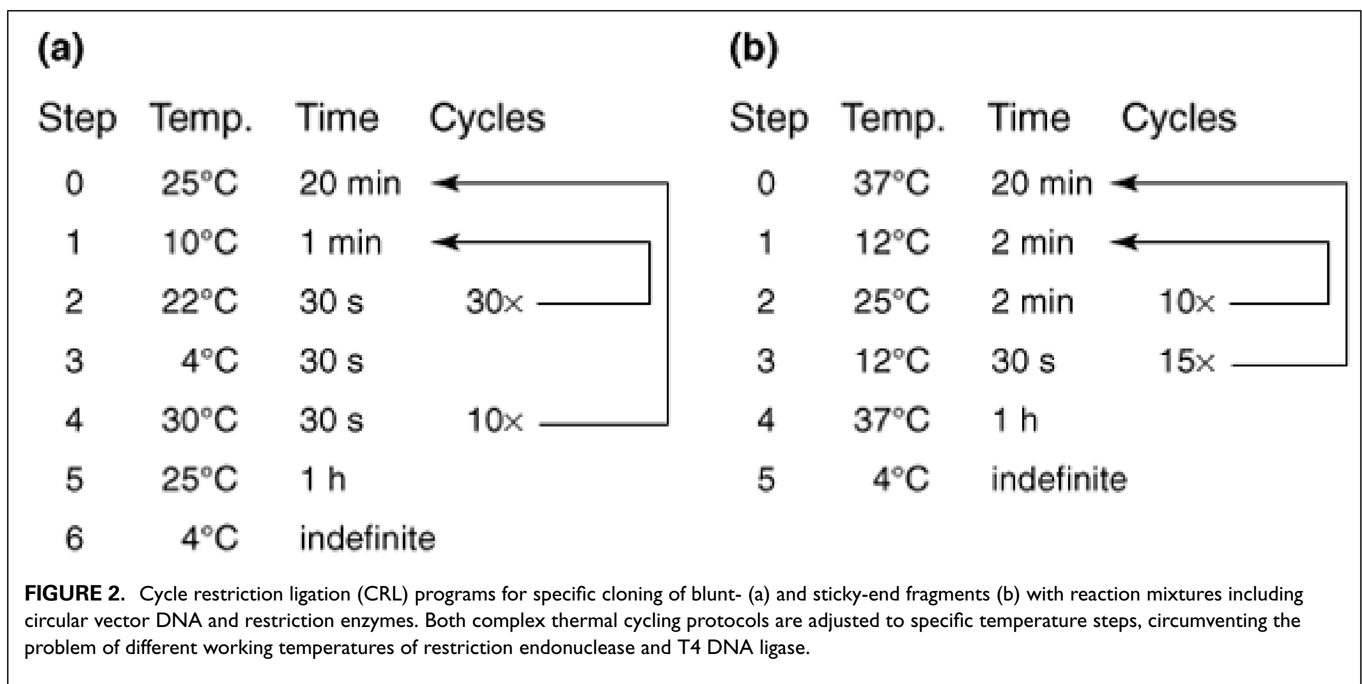
DH5α competent cells (Gibco BRL) were transformed with 5–20 ng DNA of each ligation reaction according to the manufacturer's protocol. Thirty microlitres of cell

Corresponding author: carsten.pusch@uni-tuebingen.de

1366-2120 ©1997 Elsevier Ltd. All rights reserved. PII: S1366-2120(08)70025-4

Step	Temp.	Time	Cycles
0	22°C	10 min	
1	16°C	10 min	5×
2	4°C	30 s	
3	27°C	30 s	
4	13°C	30 s	50×
5	4°C	30 s	
6	16°C	1 h	2×
7	22°C	1 h	
8	18°C	3 h	
9	4°C	indefinite	

FIGURE 1. Temperature cycle ligation (TCL) protocol alternating between ranges of suitable temperatures to enhance cloning efficiency. The cycling steps cover a wide range of temperatures to combine optimal conditions for enzymatic joining and strand annealing, thus leading to an improved yield of ligation products.



(a)

Step	Temp.	Time	Cycles
0	25°C	20 min	
1	10°C	1 min	
2	22°C	30 s	30×
3	4°C	30 s	
4	30°C	30 s	10×
5	25°C	1 h	
6	4°C	indefinite	

(b)

Step	Temp.	Time	Cycles
0	37°C	20 min	
1	12°C	2 min	
2	25°C	2 min	10×
3	12°C	30 s	15×
4	37°C	1 h	
5	4°C	indefinite	

FIGURE 2. Cycle restriction ligation (CRL) programs for specific cloning of blunt- (a) and sticky-end fragments (b) with reaction mixtures including circular vector DNA and restriction enzymes. Both complex thermal cycling protocols are adjusted to specific temperature steps, circumventing the problem of different working temperatures of restriction endonuclease and T4 DNA ligase.

suspension were spread onto LB plates containing 30 μg/ml ampicillin. As fragments containing CA repeats of various sizes [$(CA)_{9-24}$] were used for ligation, we were able to monitor the cloning efficiency by lifting the colonies onto nylon membranes (Genescreen) and subsequent hybridization of the transformed colonies with 5 pmol radiolabelled $(CA)_{15}$ oligonucleotides.

For blunt-end ligation, the transformation efficiency using our CRL increased the number of clones containing dinucleotide repeats 10−13-fold compared with standard protocols (Ref. 4) (5−13 cfu in standard methods; 53−177 cfu in CRL), whereas in sticky-end ligation (45−62 cfu in standard methods; 101−239 cfu in CRL) and in asymmetric-end ligation (70−112 cfu in standard methods; 139−522 cfu in CRL) the yield improved fourfold. Expensive chemicals like hexamminecobalt-(III)-chloride (Ref. 4) or polyethylenglycol (PEG) (Ref. 5), which are usually added to the reaction mixture as condensing agents, were replaced by the less expensive glycogen, which leads to the same effect.

The main advantage of CRL is a substantial increase in cloning efficiency, which results from continuous reopening of self-ligated vector DNA and therefore a highly efficient repression of nonrecombinant molecules. As the original restriction site is usually lost, the ligation products become stable. CRL is also easier and quicker than other methods. There is no need for time-consuming treatment of vector DNA (e.g. linearization, phenol extraction and dephosphorylation by calf intestinale phosphatase or shrimp alkaline phosphatase). As restriction enzymes are included in the cycle reaction, no self-ligated vector molecules and dimers are present after CRL. Therefore, the method is particularly valuable using vectors without the ability of blue−white selection (e.g. pBR322, pGal4). We did not observe any significant difference in cloning efficiency with respect to the fragment sizes or vector systems applied, allowing our new technique to be used in a broad range of applications.

References

1 Lund, A.H., Duch, M. and Pederson, F.S. (1996) *Nucleic Acids Res.* 24, 800–801.
2 Nizetic, D. *et al.* (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3233–3237.
3 Sealey, P.G. and Southern, E.M. (1989) Rickwood, D. and Hames, B.D. (Eds.). In Rickwood, D. and Hame, B.D. (eds) Gel Electrophoresis of Nucleic Acids, IRL Press 39–76.
4 Rusche, J.R. and Howard-Flanders, P. (1985) *Nucleic Acids Res.* 13, 1997–2008.
5 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual 2nd edn*, Cold Spring Harbor Laboratory Press.

Products Used

Pfu DNA polymerase: Pfu DNA polymerase from Stratagene
Pfu DNA polymerase: Pfu DNA polymerase from Stratagene
QIAquick-spin PCR purification kit: QIAquick-spin PCR purification kit from QIAGEN GmbH
QIAquick: QIAquick from QIAGEN GmbH
T4 DNA ligase: T4 DNA ligase from Boehringer Mannheim
ligase: ligase from Boehringer Mannheim
nylon membrane: nylon membrane from Amersham Pharmacia Biotech