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Increased cloning efficiency by temperature-cycle ligation

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Molecular cloning is a crucial, and often speed-limiting, step in many standard procedures of molecular biology. Ligation of cohesive DNA ends is normally carried out at 12–16°C to ensure a good balance between enzyme activity and stability of annealed DNA overhangs. Low temperatures generally reduce ligase activity, whereas too high temperatures may reduce cloning efficiencies by melting annealed DNA overhangs and increase overall molecular motion in the ligation reaction. Several procedures have been described to increase the efficiency ligation reactions, including the addition of condensing agents as polyethylene glycol (1) or hexamminedicobalt chloride (2). These approaches induce macromolecular crowding, and thus serve to mimic higher DNA concentrations in the ligation reaction. Other approaches seek to increase the efficiency of molecular cloning procedures by omitting the ligation step by generating long single-stranded DNA overhangs that can be annealed and transformed directly into an appropriate *Escherichia coli* host (3). Ligation of blunt-ended DNA fragments is normally carried out at room temperature using higher concentrations of T4 DNA ligase.

We have devised a simple procedure in which high enzyme activity and DNA annealing is balanced by constant temperature cycling. We find temperature-cycle ligations (TCL) may increase the efficiency of staggered cut cloning ~4–8-fold, while the efficiency of blunt-end clonings are increased ~4–6-fold (see Table 1).

The bacterial cloning vector pBluescript II KS$^+$ was digested with *Afl*III and *Hin*dIII producing two cohesive end-fragments of 434 and 2526 bp respectively, or *Pvu*II generating two blunt-end fragments of 448 and 2512 bp respectively. All enzymes were purchased from Amersham. Digested plasmid DNA was separated on 1% low-melting agarose (NuSieve) and purified as described by Sambrook *et al.* (1). For each digestion a ligation master mix was prepared containing 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM DTT, 50 µg BSA/ml, 0.5 mM ATP, 200 ng DNA and 0.1 Weiss unit T4 DNA ligase/10 µl. The purified fragments were added in equimolar amounts. The master mix was divided into 10 µl ligation reactions and either incubated at 14°C, at room temperature (22°C), or subjected to temperature cycling. Temperature-cycle ligations were carried out for 12–16 h in a Perkin-Elmer-Cetus DNA Thermal Cycler 480 programmed indefinitely to cycle between 30 s at 10°C and 30 s at 30°C. In our setting, the machine performed 10 cycles/h. Competent XL1-Blue *E.coli* cells were transformed by electrotransformation according to manufacturer's directions (BioRad), transferred to 1 ml LB medium and incubated at 37°C for 1 h. For enumeration of cloning efficiencies aliquots of 25–100 µl were spread onto LB plates containing 50 µg/ml ampicillin. The number of colonies appearing per transformation is shown in Table 1. We have tested the kinetics of temperature-cycle ligations over 12–16 h, and found them to be similar to those of ligations at constant temperatures resulting in a linear increase with time in the number of colonies appearing after transformation (data not shown). Furthermore, temperature-cycle ligation may be used together with hexamminedicobalt chloride in blunt-end ligations to give the combined effect.

Table 1. Ligation efficiencies[a]

	Ligation of cohesive ends			Ligation of blunt ends		
	14°C	TCL	TCL/14°C	22°C	TCL	TCL/–22°C
Exp. 1	1115 ± 202	4920 ± 500	4.4	55 ± 50	226 ± 35	4.1
Exp. 2	488 ± 72	3750 ± 421	7.7	25.3 ± 5.5	150.2 ± 6.2	5.9

[a]Average numbers of ampicillin resistant colonies per 1 ml LB medium ± S.D.
TCL, Temperature-cycle ligation.

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Temperature-cycle ligation is routinely used in our laboratory, and, we believe the technique to be broadly applicable in all protocols involving molecular cloning, with special relevance to difficult clonings and library construction.

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