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Y-ligation: An efficient method for ligating single-stranded DNAs and RNAs with T4 RNA ligase

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Summary

Very efficient ligation of oligodeoxyribonucleotides was attained through a simple molecular construct, which is composed of one stem and two branches (Y-shape), with use of T4 RNA ligase. Single-stranded DNAs (naturally, RNAs also) of more than 100 nucleotides (even 800 nts) were considerably ligated, approximately as theoretically expected. Owing to the molecular construct adopted, such a tiny amount of ligation products could be amplified to a sufficient amount by PCR and then recovered as single-stranded DNAs. This advantage of being amplifiable is shown to be useful for both combinatorial chemistry and evolutionary molecular engineering, which deal with a pool of diversity molecules.

T4 RNA ligase has been used for various RNA/DNA technologies due to its nature to ligate both RNAs and DNAs in single strands [1–4]. However, the ligation efficiency was not so high (a tenth of that of RNA or less) in case of single-stranded (ss) DNAs without elaborate modifications such as 3′-end ribosylation of the acceptor DNA [5,6]. This paper reports a method of high-efficiency ligation of ssDNAs using T4 RNA ligase. It is also useful for generating a diversity of molecules through combinatorial ligation as described later.

Figure 1 shows a construct used for ligation, which is composed of three parts, one stem and two branches, organized of two DNAs (5′-half and 3′-half ones). The stem part, which binds two DNAs, has a function to elevate the reaction rate because it renders intermolecular reaction into, effectively, an intramolecular one. We designate this type of ligation as *Y-ligation* after the shape of the reactants. Some of the results on the ligation efficiency performed by this method are shown in Figure 2a and the dependency of the ligation efficiency on the size of the branches is presented in Figure 2b. Evidently, oligodeoxyribonucleotides of a small size are ligated with very high efficiency (Figure 2a, lane 6), which was formerly unattainable. The larger ssDNAs ($(L_{5'} + L_{3'}) \geq 100$) were also found to be ligated in a considerable yield, of which the dependency on the size of the branches ($L_{5'} + L_{3'}$), depicted in Figure 2b, was close to the theoretical curve (Figure 2c). (Figure 2c was obtained based on a simple assumption that the ligation rate is proportional to the probability of being within a certain close distance (a) for two ends of the waving branches in order to be held together by the enzyme. The probability could be derived from the theory on random coil developed by Flory [7]. With a simplification of treating it as a Gaussian chain and with numerical treatment of the resulting equation, the theoretical curve was expressed as shown here.) The result shows that even more than 100 nucleotides of DNA can be ligated to some extent by this method and, once ligated, it can be amplified by PCR with the stem sites served for priming. This was confirmed with the successive ligations of ssDNA fragments (originated from *B. subtilis* subtilisin gene), without the modification of 3′-ribosylation of the acceptor molecule, up to more than 800 nucleotides, resulting in block-shuffled DNAs (manuscript in pre-

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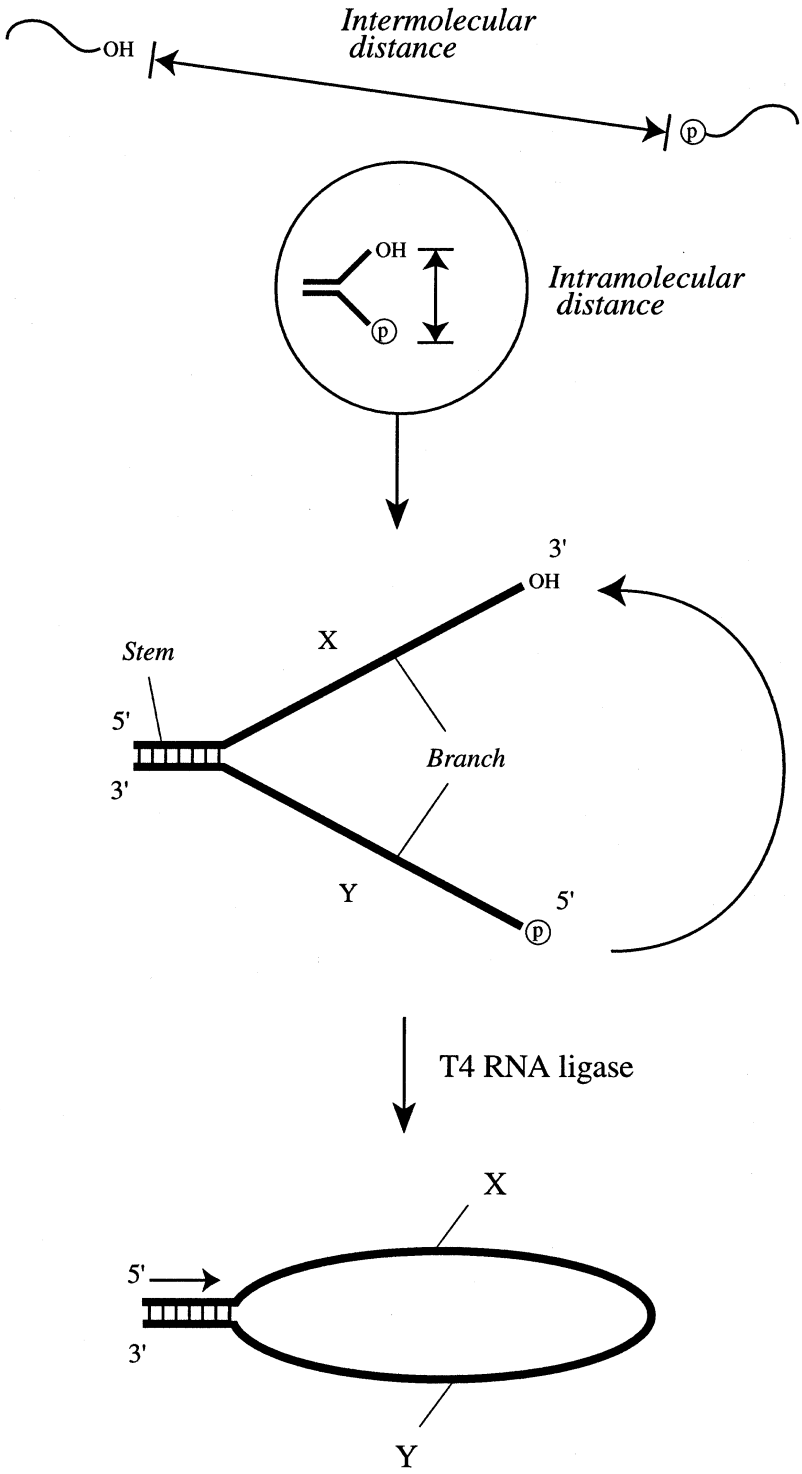

Figure 1. Schematic representation of Y-ligation. The *in-solution* distance between two substrate DNAs (an acceptor and a donor) can be converted into an intramolecular distance by way of hybridization at the stem sites. The 5′-end of the donor ('Y') should be phosphorylated and, usually, the 3′-end-most nucleotide of the acceptor ('X') is a ribonucleotide rather than a deoxyribonucleotide for the sake of ligation efficiency [5]. The stems can be utilized as primer-binding sites for PCR amplification. If necessary, the stem parts can be designed so that they can be removed with the use of restriction enzymes such as *Mbo II* and *Alw I*.

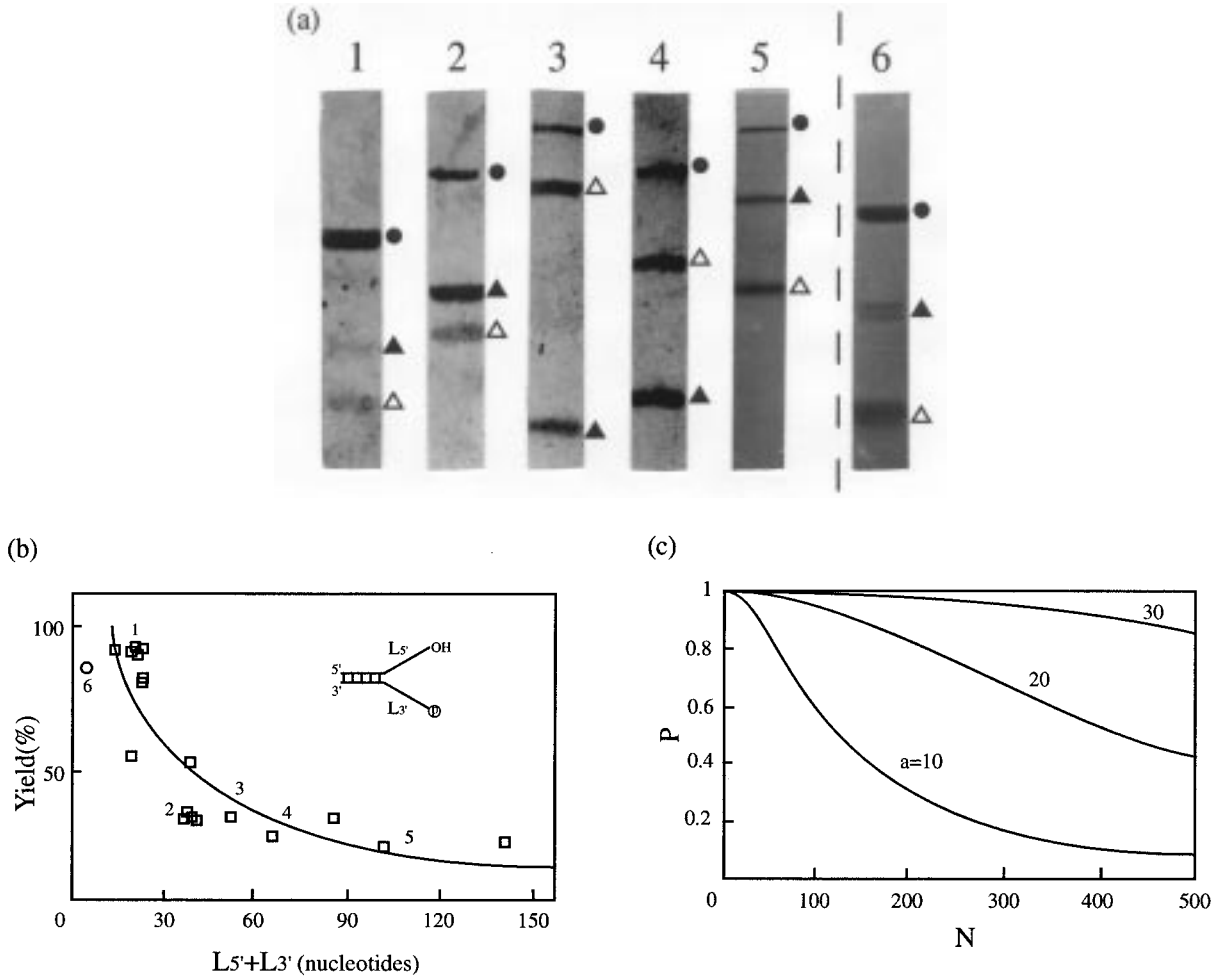

Figure 2. Dependency of ligation efficiency in Y-ligation on the size of single-stranded parts (branches). (a) Experimental results of Y-ligation. Two types of DNAs (5′-half and 3′-half) were hybridized by incubation in 50 mM Tris-HCl (pH 8.0) at 37 °C, 30 min after denaturing at 65 °C, 5 min and then ligated in a reaction buffer (50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM hexammine cobalt chloride, 1 μg/ml bovine serum albumin, 25% polyethylene glycol 6000, 0.1 mM ATP) at 25 °C, 2 h. The yields of the products were evaluated, based on relative intensity by sight from the bands obtained by gel electrophoresis (8 M urea-containing 15% polyacrylamide) and the succeeding silver staining. The DNAs used were: 1, **N**-CCCCCCc (or **N**-C_6c) (5′-half), T_{15}-**N**′ (3′-half); 2, **N**-A_{15}c , C_{20}-**N**′; 3, **N**-T_{45}c, C_6-**N**′; 4, **N**-T_{45}c, C_{20}-**N**′; 5, TACTGCGAAGAGGATCGAG-T_{33}c, AACTCGATCCGCAGATTGAAATACATTCTTCTTCGTAGATG-GATCTTCCGCT CGATCCTCTTCGCCTCGATCCTCTTCGCAGTA; 6, ACAAAATAAACAGCC, ATATGGCTGTTTATTTTGTAACTG (**N** = GCGGATCGAGTT, **N**′ = AACTCGATCCGC and c = riboC), where all sequences are written in 5′-to-3′ direction from left to right and the DNA pair for each lane represents the 5′-half (the acceptor for ligation reaction) and the 3′-half (the donor), respectively. Note that the ligation efficiency is rather high although the acceptor used for lane 6 does not contain a ribonucleotide at its 3′-end. The symbols, △, ▲, ●, represent the 5′-half, the 3′-half and ligated products, respectively. (b) Graphic representation of yield of ligation vs. branch size. The numbers 1–6 indicate the results above obtained and the additional data are also plotted. Note that error bars are not shown but these data usually contain an error (about 30% of each absolute value) due to the silver staining and evaluation-by-sight. The solid line is a fit curve drawn by hand. (c) Theoretically expected curves for the probability of ligation vs. branch size. Gaussian chains of unit length (unity) and *N* degree of polymerization were assumed for the branches of *N* nucleotides (= $L_{5'}$ + $L_{3'}$). Here, yields are supposed to be in proportion to the probability for two ends to be ligated to come close within a distance *a*. Numerical calculation is adopted (details omitted here).

paration). This nature is paramountly convenient for evolutionary molecular engineering [8] and combinatorial chemistry, since the whole set of diversity molecules can be easily amplified and recovered by PCR. It was also checked by sequencing that Y-ligation itself did not introduce any mutations, while PCR steps were confirmed to bear as many mutations as theoretically expected (data not shown).

This method exclusively has, at least, three merits besides the property of efficient ligation of DNA: (i) it can perform a ligation reaction in a low concentration of substrates since it is based on quasi-intramolecular ligation rather than intermolecular ligation; (ii) stoichiometric ligation is possible without, ideally, leaving no reactants, which is very significant for multi-step reactions; and (iii) the stem part, which is to be cleaved off after ligation, has versatile functions such as clamping together two substrates, serving as priming sites for PCR and offering cognitive sequences for further technology (e.g., block shuffling). Needless to say, ligation of RNAs and DNA/RNA hybrids (even modified nucleotide-containing RNA/DNA[9]) also, although not shown here, must be facilitated by this method.

Recently, Wang and Ruffner reported an efficient RNA ligation by T4 RNA ligase with use of an 'oligonucleotide bridge' [4], which has a common point with the method presented here: Two molecules, a donor and an acceptor, are closely tethered by hybridization mechanism. The essential difference between the two methods is in a fact that the latter requires only a single hybridization (between two substrates), while the former needs two hybridizations (between a donor/acceptor and an oligonucleotide) and an extra oligonucleotide for bridging. Technically, the simpler the better. Besides, in lower concentrations of substrates, the larger the number of components of a complex is, the less stable it becomes. This is frequently very important for such technology since reactions are often performed under the conditions of a small amount of reactants for cost reasons. The presence of extra molecules is often inhibitory, especially for PCR. Therefore, the molecular construct adopted here has enough rationale.

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