A Direct and efficient synthesis method for dumbbell-shaped linear DNA using PCR \textit{in vitro}

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ABSTRACT

A linear, covalently-closed, dumbbell-shaped DNA vector including a transcription unit is known to have both biological stability and safety and is expected to be useful for gene therapy. We established an easy, quick, and large preparative synthetic method of modified- and unmodified-dumbbell DNA using an intramolecular cyclization at the DNA termini.

INTRODUCTION

Plasmid vector-based gene transfer systems have several disadvantages for gene therapy. For example, these plasmid DNAs usually have extra genes encoding therapeutically unwanted proteins. Moreover, these extra genes contain many CpG motifs which induce immunotoxicity during gene therapy (1). To overcome these problems, a minimal-size gene transfer vector was invented (2). This new vector is a small, linear, covalently-closed, dumbbell-shaped DNA molecule that includes a transcription unit (promoter and gene of interest). The dumbbell-shaped DNA is usually generated by a ligation reaction between one transcription unit backbone and two hairpin DNAs (2,3). Using this method, however, we cannot obtain a sufficient amount of the dumbbell-DNA molecule, mainly because the ligation is an intermolecular reaction among these three independent molecules (4). To obtain a large amount of dumbbell-shaped DNA, here we invented a new strategy for gene construction \textit{in vitro} using intramolecular ligation to form the hairpin-loop structure.

![Figure 1](https://academic.oup.com/nass/article-abstract/3/1/191/1050281)

\textbf{Figure 1.} A general scheme of direct and efficient synthesis for dumbbell-shaped linear DNA using PCR \textit{in vitro}. 
RESULTS AND DISCUSSION

The key steps for the dumbbell DNA construction are shown in Figure 1. The procedure consists of three major steps.

**Step 1:** A gene of interest was amplified by PCR with a primer pair that can be modified by various substituent(s).

**Step 2:** The amplified DNA was cleaved at two site-specific positions to give two single-strand DNAs at both ends of the double-stranded gene of interest.

**Step 3:** The product was treated with T4 DNA ligase.

We emphasize that the cyclization yield (Step 3) is around ~90%, because the cyclization undergoes a simple intramolecular ligation reaction. Using this methodology, we can routinely obtain 10 μg of dumbbell DNA at one time. The dumbbell DNA with a primary amino group only in the hairpin-loop (4h) was successfully converted to a fluorescent dumbbell DNA (4c) by the reaction with fluorescein-OSu ester. In contrast, the dumbbell DNA with no primary amino group (h) did not react at all with the succinimide ester (data not shown). This means that various substituents can be site-specifically introduced only to the hairpin-loop region of the dumbbell DNA. The fluorescent dumbbell DNA (4d) was also made from the fluorescent-primer (lc) mediated synthesis.

It is known that dumbbell-shaped DNAs provide increased stability toward nucleolytic degradation by exonucleases in a biological environment because of the absence of free termini (5). The intermediate DNA (2c) and the dumbbell-shaped DNA (4c) were independently treated with exonuclease III and analyzed by an 8% polyacrylamide gel electrophoresis (PAGE). As shown in Figure 2, DNA which has the free termini (2c) totally degraded after 1 hour incubation with the nuclease, while dumbbell DNA (4c) clearly resisted against the nucleolytic degradation under the same condition.

In conclusion, we established a novel and convenient method for creating dumbbell DNA molecule with a fine yield. This PCR-mediated minimal vector construction system will open a new way for gene therapy with biological safety and stability.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** DNA stability toward exnuclease digestion. The same amount of DNA was subjected to 8% PAGE. The bands were first visualized using (a) FluorImager 595 (Molecular Dynamics). The numbered steps 2c and 4c in Figure 1 correspond to the numbers in this figure. \(λ_{ex} = 488 \text{ nm} \). The bands of approximately 600 bp correspond to fluorescein-labelled DNA. Then the gel was stained with ethidium bromide and the bands were visualized using (b) transilluminator. \(λ_{em} = 302 \text{ nm} \).

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REFERENCES AND NOTES

4. In our experiment, the ligation yield is around 40%.