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Ligation of 5' DNA Overhangs to 3' DNA Overhangs Using Short Single-Stranded Oligonucleotide Linkers

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Most methods for ligating incompatible DNA ends created by restriction endonucleases are cumbersome and unreliable. Usually, they involve the generation of blunt-ended DNA by either filling in the overhangs with a DNA polymerase or by removing the DNA overhangs with single strand-specific exonucleases (1,3). The application of these methods often results in the introduction of small deletions and a low overall cloning efficiency. This represents a serious problem for protein encoding cDNA fragments where the maintenance of the open reading frame is essential. We describe an alternative method for the ligation of 5' overhanging DNA ends to 3' overhangs that makes use of small, single-stranded oligonucleotide linkers. These oligonucleotides are complementary to the two incompatible overhangs that are to be joined. They do not contain palindromic restriction endonuclease recognition sequences and are therefore not linkers in the traditional sense (2).

In the example shown in Figure 1, this method was used to construct a chimeric open reading frame that consisted of a cDNA fragment encoding the extracellular domain of a transmembrane protein (*Drosophila* neuroglian) and a cDNA fragment encoding the transmembrane segment and the cytoplasmic domain of a different transmembrane adhesion receptor (*Drosophila* fasciclin II). Using 600 U (equivalent to 9 Weiss units) of T4 DNA Ligase (New England Biolabs, Beverly, MA, USA) with the buffer supplied by the manufacturer in a volume of 20 μ L, about 20 fmol of vector DNA were ligated with about 20 fmol of cDNA insert and 1 pmol of oligonucleotide. The reaction was incubated overnight at 15°C. Subsequently, residual ligase activity was destroyed by heat inactivation, and the ligation products were cut with *Aat*II to reduce background prior to transformation into competent *E. coli* TG-1 cells. Frozen, competent TG-1 cells were prepared using a modified calcium chloride method. Although this reaction requires the ligation of three different DNA molecules to each other, ligations containing all three components yielded several hundredfold more transformants than ligation reactions in which the oligonucleotide was missing. Plasmid DNA was isolated from selected colonies, and the success of the ligation

Figure 1. Construction of a chimeric cDNA by the linker-mediated ligation of DNA ends generated by a 5' and a 3' overhang-generating restriction endonuclease. The cDNA encoding the *Drosophila* cell adhesion molecule neuroglian contains a unique *Ava*II restriction site in front of its transmembrane segment, and the *Drosophila* fasciclin II cDNA has an *Aat*II site at an equivalent position. The reaction involved the ligation of a 3.4-kb *Not*I/*Ava*II neuroglian cDNA fragment with a *Not*I/*Aat*II DNA fragment consisting of the 3' part of the fasciclin II cDNA and the pBluescript® SK vector (Stratagene, La Jolla, CA, USA). The 5' and the 3' overhangs generated by the *Ava*II and the *Aat*II enzyme were joined with the help of a single-stranded heptaoligonucleotide (indicated in bold), which was included in the ligation reaction. This joins the neuroglian-coding sequence in-frame to the fasciclin II cDNA fragment. In general, the positions of the two restriction endonuclease sites might result in an interruption of the open reading frame, and therefore not all protein-coding DNA fragments can be ligated together in-frame using this method.

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Table 1. Pairs of Restriction Endonuclease Sites that Have Been Ligated Using Short Oligonucleotides

Restriction Sites (5'/3' Overhang)	Oligonucleotide (5'→3')	Reconstituted Restriction Sites
<i>AccI/AatII</i>	AT·ACGT	<i>AccI</i>
<i>Avall/AatII</i>	GAC·ACGT	-
<i>EcoRI/SfiI</i>	AATT·CCA	<i>EcoRI</i>
<i>NdeI/KpnI</i>	TA·GTAC	-
<i>XbaI/KpnI</i>	CTAG·GTAC	<i>KpnI</i>
<i>XhoI/KpnI</i>	TCGA·GTAC	<i>XhoI</i>
<i>XhoI/SacII</i>	TCGA·GC	<i>XhoI</i>

Pairs of 5' and 3' overhangs that have been ligated using the indicated oligonucleotide. Restriction endonuclease recognition sites that are maintained after ligation are listed in the right column.

Table 2. Influence of an Alkaline Phosphatase Treatment of the Vector DNA and of a Polynucleotide Kinase Treatment of the Linker Oligonucleotide on the Ratio of Correct to Incorrect Ligation Products

Treatment of Ligation Substrates	No. of Correct Ligation Products Per No. of Analyzed Transformants
Vector, phosphatase-treated	8/8
Vector, not phosphatase-treated	3/8
Linker, kinase-treated	4/8
Linker, not kinase-treated	7/8

Separate ligations using either alkaline phosphatase-treated or not phosphatase-treated vector DNA and either polynucleotide kinase-treated or not kinase-treated linker were performed. After transformation into competent TG-1 cells, DNA was prepared from 8 different bacterial colonies and analyzed by agarose gel electrophoresis after a digestion with appropriate restriction endonucleases.

reaction was verified by restriction endonuclease digests and DNA sequencing. Isolated DNAs from ligations without the oligonucleotide did not yield the desired plasmid product and were therefore derived from background colonies. This strategy can also be used in a bimolecular ligation reaction to introduce deletions between two 5' and 3' overhang-generating restriction sites within the same DNA construct.

In our hands, this method has worked for a number of different combinations of restriction endonuclease sites (Table 1) in different DNA vectors. With the synthesis of an appropriate oligonucleotide, it can easily be adapted to any pair of 5' and 3' over-

hang-generating restriction endonucleases. In all of the examples, which are listed in Table 1, one and sometimes both of the initial restriction endonuclease sites are destroyed. If a deleted site is absent in the anticipated product, a digestion with this enzyme after the ligation reaction can be used to reduce unwanted re-ligation products (as in the example shown in Figure 1, which includes an *AatII* digestion after the ligation reaction). As long as the initial restriction endonuclease digests are driven to completion and the vector and insert fragments are isolated by preparative agarose gel electrophoresis, it is not absolutely necessary to treat the vector with alkaline phosphatase or to phosphorylate the oligonucleotide with

polynucleotide kinase to obtain the desired ligation product (Table 2). Since the DNA overhangs involved are incompatible with each other, the number of background colonies will be low, and most transformants will contain linker-mediated ligation products. Nevertheless, in the example presented here, treatment of the vector DNA with alkaline phosphatase prior to the ligation reaction increased the fraction of correct ligation products. Only when using phosphatase-treated vector DNA and linker oligonucleotides that had not been treated with polynucleotide kinase did all of the analyzed transformants contain the correct plasmid product (Table 2).

With an available source for synthetic oligonucleotides, this strategy provides an easy and reliable alternative to traditional methods for the ligation of DNA fragments with 5' and 3' overhanging ends.

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