Site-Directed Mutagenesis Using a PCR-Based Staggered Re-annealing Method Without Restriction Enzymes


Ligation of cDNA fragments represents a pivotal reaction in recombinant DNA technology. This capability is also essential for polymerase chain reaction (PCR)-based site-directed mutagenesis, where addition or deletion of a desired sequence can be introduced into a gene of interest by using appropriately designed PCR primers (9). Generally, ligation is performed on compatible cohesive or blunt termini created by cleavage with appropriate restriction enzymes. Cohesive termini ligation is by far the method of choice since blunt-end ligation is less efficient and requires high concentrations of both DNA and ligase as well as extended reaction times (8). However, compatible ends are not always available when cDNA pieces are to be ligated. To overcome this problem, various techniques have been described for PCR-based site-directed mutagenesis (5,9), including overlap extension (5,6). In the course of a study to generate an additional insertion between two fragments of gelatinase A, we were unable to do it using the techniques mentioned above. We describe here a novel PCR-based method that enabled us to generate compatible cohesive ends for our particular experimental purposes. This approach has much broader applicability and can readily be adapted to a variety of situations involving the use of site-directed mutagenesis. The overall advantage of our approach is that it allows the investigator to introduce compatible cohesive ends at termini to be ligated, without the need for restriction enzymes.

The basic principles of the method are outlined in Figure 1. The following description provides experimental details of the procedure used to introduce a 30-bp sequence of furin substrate (depicted in bold letters TCAG in Figure 1) in the 533-bp 5′ end of the gelatinase A gene between the pro and active segments of the gene. Two different plasmids containing two different regions of gelatinase A (corresponding to regions a and b in the schematic representation).
Benchmarks

Benchmarks

tation of Figure 1) were used as a tem-
plate for the PCR. Three PCR primers for each region were used. Primers that contained the 30-bp insertion and se-
quences of the gelatinase A gene corre-
spond to number 3 (5′ P-ACGCTTC-
TGACGATTTCATACATAGACCG-
TGCCCATCTGGTGTTGC 3′) and
number 6 (5′ P-GGTCTGAGTGCTA-
GAATCGTACCAGCGTTACAA-
CTCTCTCCCTCGCAAGC 3′) in the
example shown in Figure 1. Primers
number 1 (5′ TCGAGCGCAGCTAC-
GATGGAGGCCTAAT 3′) and num-
ber 2 (5′ P-GTTGCCACATCTGGG-
TGTC 3′) are sense and antisense
oligonucleotides that amplify a 343-bp
segment of the pro segment of gelati-
nase A using plasmid pH3a as a tem-
plate. (For our experimental down-
stream use, primer number 1 was
designed to contain an additional 11 bp
that contain a NotI site; this would yield
a final product of 574 bp). Primers
number 4 (5′ CCAGCGCCAAAG-
TTGATCA 3′) and number 5 (5′ P-T-
ACAACTTCTTCCCTCGCAAGC 3′)
correspond to the 5′ activated segment
of gelatinase A and amplify a 201-bp
segment using plasmid pK-191. To
maintain the option of further amplifying
the 574-bp product with PCR, primers 2, 3, 5 and 6 were each phos-
phorylated using T4 polynucleotide ki-
nase. (If the desired product is to be lig-
ated to a plasmid, it is sufficient to
phosphorylate only one of the two
pairs.)

PCRs were performed in a final vol-
ume of 50 µL. A typical reaction con-
tained 1× Pfu reaction buffer, dNTPs at
250 µM each (Pharmacia Biotech, Uppsala, Sweden), primers at 1 µM
each (HSC/Pharmacia Biotech, Tor-
onto, ON, Canada), 10 ng of plasmid
dNA, and 2.5 U of Pfu DNA Poly-
merase (Stratagene, La Jolla, CA,
USA). Reaction tubes were heated to
95°C for 5 min and then cycled 40× at
95°C for 30 s, 55°C for 30 s and 72°C
for 2 min/kbp. At the end of the run,
tubes were incubated at 72°C for 7 min.
Products were resolved on 1% agarose
gels containing 0.5 µg/mL of ethidium
bromide. The PCRs yielded four prod-
ucts, two for each region, that were

Figure 2. Agarose gels of PCR and ligation products generated for staggered re-annealing site-di-
rected mutagenesis. (Panel A) PCR products generated with primers 4 and 5 (lane 2), primers 1 and 2
(lane 3), primers 4 and 6 (lane 4) and primers 1 and 3 (lane 5). PCR products depicted in lanes 2 and 4
were pooled and those in lanes 3 and 5 were pooled, melted and re-annealed. Arrowheads indicate migra-
tion of PCR products. (Panel B) PCR products, after staggered re-annealing corresponding to the 5′
region of the amplified segment (region a in Figure 1; lane 4) and the 3′ region of the amplified segment (re-
gion b in Figure 1; lane 5), were combined and ligated for 1 h at room temperature. Ligation mixture
was resolved in lane 6, and the ligated 574-bp product (arrow) as well as the unligated blunt-ended products
(arrowheads) is apparent. In parallel, 2 blunt-ended products before staggered re-annealing were ligated.
No ligation product is evident (lane 3). Products before blunt-end ligation are shown in lane 2. (Panel C)
PCR using primers 1 and 4 (lane 2) and primers 7 and 4 (lane 3). Lane 1 in all panels is the 100-bp ladder.
varied in 30-bp segments from each other (Figure 2, Panel A). The two tubes for each region were combined, and an equal volume of redistilled formamide (Life Technologies, Gaithersburg, MD, USA) was added to yield a final concentration of 50%. Although the melting procedure is carried out under conditions that are not optimal for Pfu DNA polymerase, to avoid any potential filling of the 5′ overhangs by Pfu DNA polymerase during this process, EDTA was added to a final concentration of 1 mM. Samples were heated to 98°C for 5 min to facilitate melting of the strands and then cooled to 22°C for 5 min to allow re-annealing. At this stage, we expect four products from each region, two original and two newly created, containing 30-bp cohesive termini as 5′ or 3′ overhangs (see Figure 1). The cohesive termini for each of the 363-bp and 201-bp regions to be ligated create compatible ends that ultimately give rise to one final product containing the 30-bp insert. After melting, the cDNA mixture of each region was purified separately (QIAquick™ PCR Purification Kit; Qiagen, Chatsworth, CA, USA). The two regions were then combined and ligated (0.1 U of T4 ligase, 1 h at room temperature) to yield the predicted 574-bp product (Figure 2, Panel B, lanes 4–6). At the same time, a control ligation was performed in parallel utilizing the phosphorylated 343-bp and the 201-bp 5′ blunt-ended products. No blunt-end ligation was evident (Figure 2, panel B, lanes 2 and 3), suggesting that successful ligation of the cohesive termini had taken place with formation of the desired 574-bp product.

Next, the 574-bp band was gel-isolated (QIAquick Gel Extraction Kit; Qiagen) and subjected to PCR using the outer primers labeled numbers 1 and 4 in Figure 1. As seen in Figure 2, several major products other than the expected 574-bp product were generated (Figure 2, Panel C, lane 2), explaining why the trial to generate the 574-bp product using the overlap extension was unsuccessful.

To verify that the final 574-bp product generated is indeed the desired one, the matching sense primer number 7 (5′ CAATACCTGACACCCTT 3′), that should yield a 405-bp product with primer number 4, was used for PCR amplification. Inspection of Figure 2 shows that a 405-bp product was generated using the ligation reaction product as a template (Figure 2, Panel C, lane 3). A similar result was obtained when the 574-bp ligation product was first gel-isolated and then used as a PCR template (data not shown). To verify the identity of the 574-bp product, it was sequenced in the antisense orientation using fluorescein isothiocyanate (FITC)-labeled primer number 8 (5′ TGTCGCTCTCCATCATGG 3′) as previously described (1). The sequencing yielded a 329-bp cDNA containing the 30-bp furin substrate between the pro and active segments of gelatinase A. This sequencing data proves that the ligation product resulted from staggered re-annealing ligation rather than blunt-end ligation of regions a and b. Note that in this study, the 574-bp ligation product was gel-isolated at the final step since the target cDNAs were inserted in plasmid that had considerably higher molecular weight than the product. When target DNA has similar molecular weight to the mutated products, the two staggered re-annealing products should be separated from the original DNA by gel-isolation before ligation.

We have described a novel PCR-based approach for the introduction of cohesive termini into a given sequence without the use of restriction enzymes. This method can be used for addition of one or more bases of cohesive termini into a sequence. Also, it can be used for creation of new restriction sites for ligation into a plasmid in the region flanking the amplified piece. We were able to clone a 515-bp RNase H cDNA into Ncol sites of a plasmid using the principles of the staggered re-annealing method described in this communication (2). This method could also be used in site-directed mutagenesis for single-site addition or for addition (as in this study) or deletion of any sequence into genes under conditions compatible with PCRs. The procedure described here has obvious advantages compared to blunt-end ligation and offers an alternative approach to the overlap extension method. In addition, it utilizes only one PCR while the overlap extension uses two reactions overall.

Consequently, the potential of error insertion to the amplified product is reduced (4). Note that Pfu DNA polymerase is an essential requirement in our method because this enzyme does not create non-template 3′ overhangs as do other heat-stable DNA polymerases like Taq polymerase (3,7). Finally, it is worth commenting on a potential experimental limitation of the method. Since this technique is ultimately dependent on efficient melting of DNA strands, its usefulness may gradually decline when creation of cohesive termini in cDNA longer than 500 bp is required. However, future development of improved melting protocols could overcome this limitation and enable splicing of longer DNA segments.

REFERENCES
Semiquantitative RT-PCR: Enhancement of Assay Accuracy and Reproducibility


Reverse transcription-polymerase chain reaction (RT-PCR) has been widely accepted as a highly sensitive and specific method, particularly for analysis of rare transcripts in small amounts of tissue or cultured cells. While detection of such transcripts is a relatively easy task, accurate quantification of specimen transcript content is much more difficult. Although variability due to factors such as salt or primer concentration and annealing temperature may be controlled (2,4), tube-to-tube variation is often encountered, and the cause is not readily apparent. For example, others have shown (1) that PCR analysis of a single sample prepared as a master mixture and analyzed in replicate in the same amplification protocol may yield results for individual replicates that differ by as many as six fold.

Alternate RT-PCR protocols have been developed to minimize tube-to-tube variability that influences assay reproducibility. These include co-amplification of exogenous or endogenous control sequences to correct for variable product yields due to differences in individual RT or PCR efficiencies (2,4). For example, semiquantitative RT-PCR involves co-amplification of the target sequence and an endogenous control sequence whose content is not altered by the experimental manipulation. Target sequence yield is subsequently normalized to control sequence yield by using measurements obtained during the exponential phase of amplification. This study was supported by the Kidney Foundation of Canada and the Medical Research Council of Canada as part of the Membrane Biology Group. Address correspondence to Mel Silverman, Medical Science Building, Room 7207, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

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