

PCR using a thermostable polymerase with 3' to 5' exonuclease activity generates blunt products suitable for direct cloning

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The cloning of polymerase chain reaction (PCR) products provides one with a stable form of the amplified segment with restriction enzyme sites or other sequences useful in subsequent studies flanking the inserts. Blunt cloning is often an attractive approach but is frequently unsuccessful due to the template-independent addition of a nucleotide to the 3' end of one or both strands (1). This problem can be solved by 'polishing' the ends using the Klenow fragment of DNA polymerase I which contains 3' to 5' exonuclease activity (2, 3). We hypothesized that use of a thermostable DNA polymerase with intrinsic 3' to 5' exonuclease activity in the PCR reaction would yield products with blunt ends that would clone more efficiently into a blunt-cut vector. To test this hypothesis we performed amplifications using two types of polymerases and compared the cloning efficiencies of the resulting products.

We chose to compare Taq DNA Polymerase (AmpliTaq™, Perkin-Elmer Cetus) with Vent DNA Polymerase (Vent_R™, New England Biolabs), which possesses 3' to 5' exonuclease activity (4). A 213 bp fragment was amplified from the pBH10 clone of HIV-1 (5) using two units of either polymerase per 100 μl reaction. A portion of the Taq produced PCR products were treated with a 'polishing' procedure (2). All of the products were then extracted with phenol/chloroform, precipitated in the presence of ethanol and resuspended in ddH₂O (6). These procedures yielded three types of products referred to as Taq, Taq-polished, and Vent products.

A portion of each product was treated with T4 DNA Kinase (Promega). Ten ng of each, with or without Kinase treatment, was then ligated into 100 ng of SmaI-digested pBluescript II (Stratagene) previously treated with calf intestinal phosphatase (BMB). Vector ligated in the absence of insert served as a background control. One half of each ligation was transformed into the TB-1 strain of *E. coli* and subsequently plated on agar plates containing IPTG and X-gal using standard procedures (6). The percentage of white colonies was determined and a PCR colony screening technique employing primers flanking the cloning site was used to verify presence of insert in selected white colonies (7).

To determine if the various PCR products were blunt and flush with the ends of the primers, a separate ligation and recutting analysis was performed. The primers used in the original PCR contained three G's at their 5' termini and thus were expected to generate novel SmaI sites following successful concatamerization (8). To examine this, the various products

treated with T4 DNA Kinase were self-ligated. A portion was then digested with SmaI enzyme (Promega). All samples were then analyzed by gel electrophoresis.

These studies showed that Taq-polished and Vent-amplified products treated with T4 Kinase yielded 49% and 53% white colonies respectively, against a background of 10%. No other groups exhibited a percentage of white colonies substantially above background.

The Taq-polished and Vent groups also appeared comparable upon insert analysis by PCR with correct size inserts observed in 4/5 and 3/4 transformants amplified, respectively. No inserts were observed in other groups examined. Separate ligation analysis showed that approximately 65% of the products of these two groups successfully ligated into high molecular weight forms. Upon treatment with SmaI, the majority of this DNA was digested to completion.

These results support our hypothesis that a thermostable polymerase with 3' to 5' exonuclease activity yields PCR products with ends that are both blunt and flush with the ends of the primer sequence. We demonstrate that such ends are suitable for efficient and accurate ligation into a blunt-cut vector.

This technique does not require 'polishing' of PCR products, nor does it require the additional vector preparation inherent to 'T'-cloning strategies (9, 10). It may therefore represent the strategy-of-choice for the non-directional cloning of PCR products.

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