Demonstration of the Expand™ PCR System’s Greater Fidelity and Higher Yields with a lacI-based PCR Fidelity Assay

Thermostable polymerases possessing a 3’→5’ exonuclease, or “proofreading,” activity are often used in polymerase chain reactions because they offer higher fidelity amplification than enzymes lacking this activity. Unfortunately, these proofreading enzymes also produce substantially lower amplification yields than polymerases without proofreading activity. On the other hand, “non-proofreading” DNA polymerases (e.g., Taq DNA polymerase, Tth DNA polymerase) lack the high fidelity required for applications in which accurate replication is placed at a premium. Coupled with a recent breakthrough in PCR enzyme technology (1), these facts have led to the development of the Boehringer Mannheim Expand™ PCR System, optimized mixtures of Taq DNA polymerase and the proofreading enzyme Pwo DNA polymerase.

We wanted to demonstrate that the Expand PCR System offers a better combination of high yields and high fidelity amplification than non-proofreading enzymes (e.g., Taq DNA polymerase) or proofreading enzymes. Therefore, we performed a PCR fidelity assay (Figure 1) based on the amplification, circularization, and transformation of the pUC19 derivative pUCIQ17, which contains a functional lacIq allele (1). PCR-derived mutations in lac result in a de-repression of the expression of lacZα and subsequent formation of a functional α-galactosidase enzyme, which can be easily detected on X-Gal indicator plates.

Materials and Methods

Construction of pUCIQ17

The truncated lacI gene of pUC19 was substituted by a functional copy of lacIq. A 178 bp Pvu II-Afl III fragment was replaced by a 1121 bp DNA fragment encoding lacIq (Figure 2). The α-complementing E. coli strain DH5α (2), once transformed with the resulting plasmid pUCIQ17 (3632 bp), produces white (LACI') colonies on LB plates containing ampicillin (100 μg/ml) and X-Gal (0.004% w/v).

Template preparation

pUCIQ17 was linearized by digestion with Dra II. A typical PCR reaction contained 5 or 10 ng of linearized, gel-purified plasmid DNA.

PCR primers

Both PCR primers used have Clai cleavage sites at their 5’ ends:

- Oligonucleotide CLA33 (34-mer, 24 matches: 5’-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3’), primes “left.”
- Oligonucleotide CLA55 (36-mer, 26 matches: 5’-AGCTTATCGATAAGCGATGCCCAGGAGCAAGAAGC-3’) primes “right” to the Dra II site of pUCIQ17 (pUC19). The length of the resulting PCR product is 3,493 bp.

Polymerase chain reactions

The polymerase chain reactions were performed according to the conditions detailed in the manufacturers’ instructions. We used the following cycling conditions for all DNA polymerase preparations except the Expand PCR System:

- Denaturation: 10 s at 94°C
- Annealing: 30 s at 57°C
- Extension: 4 min at 72°C for 18 cycles.

The following cycling conditions were employed for the Expand PCR System:

- Denaturation: 10 s at 92°C
- Annealing: 30 s at 57°C
- Extension: 4 min at 68°C for 18 cycles.

Purification and circularization of the 3.5 kb PCR products

After the PCR, the amplification products were PEG-precipitated (without previous phenol treatment) according to Barnes (3). After a restriction digest with Clai, the DNA was purified by gel electrophoresis.
Ligation reactions were carried out with the Boehringer Mannheim Rapid Ligation Kit; DNA concentrations per ligation reaction did not exceed 30 ng.

**lacI-based assay**

The resulting PCR-derived plasmids were transformed in E. coli DH5α as described by Hanahan (2), and plated on LB Amp100 X-Gal plates. After incubation overnight at 37 °C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation published by Keohavong and Thilly (4):

\[
f = \frac{-\ln F}{d \times b}
\]

where \(F\) is the fraction of white colonies:

\[
F = \frac{\text{white (LACI+) \text{total colony number}}}{d = \text{number of DNA duplications}}
\]

and \(b\) is the effective target size of the (1080 bp) lacI gene, which is 349 bp according to Provost et al. (5); there are 349 phenotypically identified (by color screening) single-base substitutions (nonsense and mis-sense) at 179 codons (approximately 50% of the coding region) within the lacI gene (5). Frameshift errors, which may occur at every position in the 1080 bp open reading frame of lacI, are not taken into account because little information is available for the specific DNA polymerases used in PCR systems (except for Taq DNA polymerase, which has a frameshift error rate of about 1/106 of the base substitution error rate determined in a one-pass assay [6,7]).

**Religation control**

Fifty nanograms of Dral II-linearized, gel-purified pUCIQ17 DNA was religated, and an aliquot of the ligation reaction was transformed into DH5α. After incubation overnight, only 1 of 3750 growing colonies (0.027%) showed a blue (LACI+) phenotype on LB X-Gal plates; therefore, formation of concatameric ligation products (with subsequent intramolecular recombination in E. coli that eliminates an additional origin of replication) seems to be a very rare event. Restriction analysis of PCR-derived plasmids isolated from blue colonies also confirmed that the LACI+ phenotype originates in PCR-derived mutations of lacI, but not in deleterious recombination events after transformation of the ligated DNA in DH5α.

**Results**

Table 1 illustrates that Boehringer Mannheim's Expand PCR System mixtures offer better combinations of high amplification yield and high fidelity PCR. They feature lower error rates than the enzymes lacking proofreading activity, such as the Taq DNA polymerase preparations and Tth DNA polymerase. In addition, the Expand PCR System mixtures produce substantially higher yields than all proofreading DNA polymerases, even from smaller amounts of DNA template. All other commercially available polymerase mixtures tested thus far demonstrated error rates in the range of Taq DNA polymerase, except the variation has been between 2.9 x 10⁻⁵ and 2.1 x 10⁻⁵ (results not shown; publication in preparation).

The ligation-based fidelity system described in this article represents a rapid, convenient method for evaluating error rates for DNA polymerases in PCR systems. Due to low background activity, it is possible to measure error rates in the range of 10⁻⁴ to 10⁻⁶. The calculated error rate for Taq DNA polymerase is in good agreement with the published value (8).

**Table 1. Fidelity of different DNA polymerases in PCR.** Enzyme preparations marked with a “1” are mixtures of two polymerases, one of which possesses proofreading activity and one of which lacks proofreading activity. Enzyme preparations marked with a “2” are single enzymes possessing proofreading activity. Enzyme preparations marked with a “3” are single enzymes lacking proofreading activity.

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>Template amount [ng]</th>
<th>Yield [ng]</th>
<th>DNA duplications (d)</th>
<th>blue colonies LACI+</th>
<th>white colonies LACI+</th>
<th>total number of colonies</th>
<th>% LACI</th>
<th>Error rate (ER)</th>
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<tbody>
<tr>
<td>Expand™ Long Template PCR System™</td>
<td>5</td>
<td>3000</td>
<td>9.2</td>
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<td>73</td>
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<td>2768</td>
<td>2.6</td>
<td>8.3x10⁻⁶</td>
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<td>1500</td>
<td>8.2</td>
<td>167</td>
<td>2202</td>
<td>2369</td>
<td>7.1</td>
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<td>8.2</td>
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<td>7.2</td>
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<td>2617</td>
<td>3.4</td>
<td>2.2x10⁻⁵</td>
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References