to be insert length-dependent: both exon 12 and exon 13 are about 370 bp in length, yet exon 12 had a mutation rate of 70.6% and exon 13 of only 23.5%.

Most of the hMSH2 mutations generated by our method were single substitutions including transversions and transitions. All twelve possible substitutions were obtained. The A→G and T→C mutation appeared to predominate (Table 2). The combination of these two totaled 63.2% of all mutations. The G→C and C→G mutation was the least seen and only comprised 1.4% of all mutations. Similar results were also reported (3,5). The A→G and T→C mutation was more frequent because of favored G:T wobble pairs and could be reduced by lowering the dATP and dGTP concentration during PCR mutagenesis.

A total of 278 independent single substitutions were distributed across 147 clones. The number of substitutions per clone did not follow a Poisson distribution. The majority (53.5%) of all mutants in this study contained a single substitution per clone. Next, 25.2% of them had two independent substitutions within the same cloned insert. Only 10.7% carried 3 independent substitutions, and less than 6.3% of them contained 4 or more independent substitutions. There were very few deletion mutations found (about 4.4%) with this procedure. Fromant et al. (5) have also reported an in vitro mutagenesis procedure using PCR and manganese. Our procedure differs significantly from that we used half the manganese ion concentration, one quarter the magnesium ion concentration, 1/170 the dNTP concentration and PCR cycles of significantly shorter duration. On the average, they reported multiple substitutions per target DNA, while our procedure resulted in only one substitution per clone in approximately half of the mutated clones. These mutants containing a single substitution are ideal for studying the effect of amino acid changes on gene function.

This effective random mutagenesis method has several advantages over existing methods: it uses a one-step PCR mutagenesis and a one-step cloning procedure, achieves a high mutation

rate and generates a large number of mutants containing a single substitution per clone.

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Near-Zero Background Cloning of PCR Products

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Cloning of polymerase chain reaction (PCR)-amplified fragments has become a widely used technique in molecular biology. Common cloning strategies include: sticky-end cloning, blunt-end cloning, TA cloning and ligation-free cloning (1,5). Among these, sticky-end cloning is the most efficient and is preferred whenever possible, especially for subcloning.

In sticky-end cloning, restriction sites are designed into the two PCR primers (15). Often the two sites are different to allow directional cloning. After amplification, the PCR fragment and the vector plasmid are digested with restriction enzymes, purified, ligated and transformed into *E. coli* (13). Since self-ligated and uncut vector plasmid can also transform bacteria, many colonies with no insert (false-positives) are generated as background, and a large number of colonies have to be screened to find a colony with an insert (true-positive). For this reason, several strategies have been developed to reduce or eliminate the background caused by self-ligated or uncut vector. For example, the linear vector can be dephosphorylated before mixing with PCR product and ligation. However, this not only reduces the cloning efficiency (13) but also is tedious since the PCR product and the vector have to be digested and purified separately before mixing the PCR fragment and the dephosphorylated vector for ligation. Alternatively, positive-selection vectors have been developed to reduce false-positive colonies (2-4,6,7,9,10,12,14,18,19), but these "zero-background" approaches require special vectors and, very often, special strains of bacteria (12,14,18).

Earlier studies have found that blunt-end PCR product cloning efficiency could be improved by digestion of the ligation product with appropriate restriction enzymes during (11) or after ligation (16). Moving beyond this strategy, we developed a simple and universal sticky-end cloning strategy in which we use a restriction digestion step between ligation and transformation,

Benchmarks

Table 1. Protocol for Near-Zero Background Cloning of PCR Products

1. Design two restriction cloning sites into the two PCR primers. Choose the two cloning sites such that they flank at least one unique site in the polylinker region of the vector. For efficient digestion, also provide enough overhang bases beyond the cloning sites on the primers (8).
2. Perform PCR amplification to obtain a DNA fragment.
3. Mix the PCR product with vector plasmid. Use as much excess vector plasmid as is practical.
4. Digest the PCR fragment/vector plasmid mixture with the two restriction cloning enzymes in an appropriate buffer.
5. Clean up the mixture by phenol/chloroform extraction and ethanol precipitation.
6. Ligate the PCR/vector mixture with T4 DNA ligase (e.g., at 16°C for 16 h).
7. Inactivate the ligase by incubating at 70°C for 10 min.
8. Mix the ligation product with 5 vol of sterile water to dilute the ligation buffer.
9. Digest the ligation product with at least one selection enzyme in an appropriate buffer. The selection enzyme(s) should cut in the vector between the two cloning sites but not in any other locations on the vector or in the insert.
10. Transform *E. coli* cells by electroporation.
11. Pick colonies and screen for insert.

resulting in greatly reduced background colonies. Specifically, for each cloning project, we choose two restriction enzymes (selection enzymes) to digest the ligation product before transforming into bacteria (Figure 1). The two selection enzymes chosen cut only between the two restriction cloning sites in the vector's polylinker region, which would be replaced if the insert had been cloned. Of course, the two selection enzymes must also not cleave the insert DNA. This digestion step linearizes any self-ligated or undigested vector, but the plasmids with an insert remain circular. Since circular DNA transforms bacteria much more efficiently than linear DNA (13,16), nearly 100% of colonies recovered contained an insert. For convenience, we refer to this scheme as "near-zero background cloning". The details of the protocol are given in Table 1.

A key step of the near-zero background cloning protocol is the digestion of the ligation product before transformation. Since T4 DNA ligase and the selection enzymes often require different buffer conditions, the cloning efficiency would be greatly diminished if the ligation product were digested directly with selection enzymes. Removing the buffer components of the ligation mixture (e.g., by phenol/chloroform extraction followed by ethanol precipitation) could solve this problem, but this adds an extra purification step. We eliminated the interfering

effect of ligation buffer by simply diluting the ligation mixture 5-fold with sterile water before adding the restriction digestion buffer and the selection enzymes. The two selection enzymes may also require different restriction buffers. Thus, the restriction buffer should be chosen such that both enzymes have at least 75% of the maximum efficiencies in the preferred buffers. These steps keep the selection step simple but efficient.

Following the digestion of the ligation product, most electroporation protocols recommend purification of the ligation/digestion product to remove salts in the restriction buffer to prevent arcing. However, the extremely high efficiency of electroporation transformation makes it possible to use a very small amount of ligation product directly for electroporation. We routinely use 0.5–1 µL ligation/digestion product for electroporation, and we have not experienced any arcing even with high-salt restriction buffers. Eliminating two purification steps before transformation greatly simplifies the process and reduces the hands-on time of the cloning scheme.

We observed that using only one selection enzyme can increase the percentage of true-positive colonies to 80%–100%, and higher PCR product/vector molar ratios (>10) can also improve the percentage of true-positive colonies to approximately 80%. How-

Benchmarks

ever, digesting the ligation product with two selection enzymes before transformation consistently yielded the highest percentages (often 100%) of colonies with the correct insert.

We verified the efficiency of the near-zero background cloning scheme in our studies of the bacterial mercury-resistance locus *mer* (17). We cloned *merR* (wild-type, 4 point mutants and 2 truncated forms) and *merB* (organomercurial lyase) into pET-21b(+) (Novagen, Madison, WI, USA) for overexpression and purification of C-terminal 6xHis-tagged proteins. After digestion of the ligation product and transformation into

E. coli strain DH5 α TM (Life Technologies, Gaithersburg, MD, USA), we used plasmid miniprep and restriction mapping to screen for the inserts. We found the expected insert in all of 51 colonies for 8 different plasmids. The success of cloning was further confirmed by dideoxyribonucleotide sequencing of 16 and electrospray mass spectrometry of 6 overexpressed proteins.

Compared with many other PCR cloning strategies, the near-zero background cloning scheme is compatible with most cloning vectors and does not require lengthy manipulations (such as dephosphorylation by alkaline phos-

phatase), exotic restriction enzymes or reagents, special vectors or special *E. coli* strains. Thus, without major modifications, the near-zero background cloning scheme could be applied in many cloning projects.

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Figure 1. Near-zero background cloning scheme. The vector has cloning sites for restriction enzymes Y and Z, which flank the two sites for the selection enzymes R and S. The two cloning sites for Y and Z are also designed into the two PCR primers P1 and P2, respectively. PCR amplification yields a product with the two cloning sites near the two ends. The PCR product is then mixed with the cloning vector and digested with cloning enzymes Y and Z. After purification by phenol/chloroform extraction and ethanol precipitation, the mixture is ligated, forming two types of circular plasmids: those with an insert and those that self-ligated or were uncut. Further digestion of the diluted ligation product with the selection enzymes R and S cleaves the vector to a linear form, while the plasmids with an insert remain circular. Since only the circular plasmid with an insert can transform *E. coli* efficiently, the colonies from the self-ligated or uncut vectors are greatly reduced, resulting in near-zero background colonies.

Benchmarks

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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.