

Student Protocol

Introduction

In this experiment, a portion of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene will be cloned. Because it is a vital metabolic enzyme involved in glycolysis, one of the most basic of biological processes, the GAPDH protein is highly conserved between organisms, especially in vital domains of the enzyme, such as the active site. However, this does not mean that the gene and DNA sequence are identical between organisms. A large percentage of a gene's DNA does not code for protein — this "intronic" DNA is not subject to the same selective pressures as DNA that does code for protein. In addition, in the gene sections that do encode protein (the exons), there is degeneracy of the genetic code, which means that multiple DNA triplet codons encode the same amino acid. (For example, GCA, GCC, GCG, and GCT all code for alanine.) Also, some regions of the protein less vital to enzyme function do not have the same degree of selective pressure as the regions that are essential to enzyme function, such as the enzyme active sites or cofactor binding sites.

To clone a known gene from a less well-characterized organism, primers must be designed that are complimentary to conserved regions of the GAPDH gene. However, even conserved regions are not identical between organisms, so a best guess of the gene sequence is made using a comparison alignment of the sequences of the gene from different, related organisms, with the understanding that the primers will not be an exact match to the sequence and may amplify non-specific sections of DNA in addition to the target sequence. A second set of primers is then designed, interior to the first set of primers, and used to amplify the PCR products from the first round of PCR. This is called nested PCR. Nested PCR works because there is an extremely slim chance that any DNA that was amplified non-specifically in the first round of PCR will also contain these interior sequences, while the specifically amplified DNA (the target sequence) should contain these sequences, even if the match is not perfect.

The Experiment

The strategy for this experiment uses nested PCR to amplify portions of the GAPC gene from genomic DNA (gDNA) of the plant of interest. In the initial round of PCR, a set of primers using degenerate (less specific) sequences will amplify the GAPC gene from genomic DNA. Then, in the second round of PCR (the nested PCR), a more specific set of primers will amplify GAPC from the initial PCR products. The primers are color-coded; the initial PCR primers are blue and the nested PCR primers are yellow. It is very important not to reverse the order in which the primers are used or to mix the two primer sets together in the PCR reactions.

Before performing the nested PCR, the primers that were not incorporated into PCR products in the first round must be removed so that they do not amplify target DNA in the second round of PCR. To remove the primers, exonuclease I, an enzyme that specifically digests single-stranded DNA, will be added to the PCR products from the first round. After exonuclease I digests the primers, the enzyme must be inactivated to prevent the exonuclease from digesting the nested primers that will be added for the next round of PCR.

Following exonuclease treatment and inactivation of the enzyme, the PCR products from the gDNA template(s) generated in the first round of PCR need to be diluted. The products from the first round of PCR contain both the original template gDNA and the PCR products, GAPC-like sequences. The GAPC-like sequences predominate, so they are most likely to be amplified during nested PCR, but diluting the gDNA makes it even less likely that it will be a template as well.

As each PCR reaction takes approximately 3–4 hours to run, it is most practical to run the PCR reactions on separate days. Since the reagents used in these experiments function optimally when prepared fresh, it is highly recommended that the reagents be prepared just prior to setting up the reactions and running the PCR.

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Didier Musso



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