Outline of protocol for sequencing PCR products

from Howard Judelson 5/1/02

PCR

1. Perform PCR reaction, in 25 µl (typically).

2. Run 1/2 of reaction on TBE gel to assess amplification products.

Sequencing directly from PCR products

1. This is feasible when the PCR reaction has yielded only 1 major band. An advantage of sequencing directly from the PCR products is that occasional errors in base incorporation will not be detected, i.e. they will be diluted out by the "correct" sequence.

2. Purify DNA using Wizard DNA Clean-up kit (Promega) or Qiaquick PCR purification kit (Qiagen). Both work fine; I prefer the Wizard kit due to slightly higher yields (60-90% recovery is typical). Follow the kit's instructions, purifying the DNA in a minimum volume.

3. Quantitate the recovered DNA. Run a small amount (hopefully about 20 ng, or 2-3 µl of the DNA depending on the original PCR reaction) on a TBE gel (containing ethidium bromide) along with a standard. A useful standard is a linearized plasmid, lambda DNA, or a previously purified sample. Run 0, 10, 30, and 100 ng of the standard. Run the tracking dye 1 cm or less. Eyeball guestimate the DNA concentration in the unknown.

4. Aliquot DNA and primer for sequencing. The attached sheet shows the recommendations for template and primer quantity from the UCR facility. It is important not to add too much DNA for sequencing. Less is better.

At UCR, they ask that samples be provided in a 0.2 ml thin-wall flat-top PCR tube.

Slab-gel instrument facilities (like at Arizona State University, which use a ABI377) require 2-3 x more DNA per reaction.

Sequencing from cloned PCR products

1. This often yields higher-quality sequences than PCR products in automated sequencing reactions. However, due to the possibility of errors by Taq polymerase, it may be necessary to sequence more than one clone to be sure of the "consensus."

2. Clone the PCR product using the pGEMT-EZ kit (from Promega). This takes advantage of the T overhang produced by Taq polymerase. Follow the kit's instructions.

Typically, I take 1-2 µl of the PCR product (unpurified from the PCR reaction is OK) per ligation.

3. Transform the ligation into competent cells. These can be prepared in the lab or purchased from any of the standard vectors. Some E. coli cells yield DNA that doesn't sequence well; we use DH5-alpha, which is commonly available.

If you use chemically competent cells (Ca^{+2}-treated): take 5 µl ligation, place in 1.5 ml microfuge tube ON ICE. Add 100 µl cells ON ICE. Leave ON ICE 30 minutes. Heat shock at 37C for 5 min (or 42 C for 30 sec). Add 1 ml LB (or even better, SOC) media and wait 45 minutes.

4. Plate the transformed cells on LB agar (1.5%) plates containing 75 µg/ml ampicillin, XGAL, and IPTG.

IPTG: isopropyl thiogalactoside, or isopropyl beta-D-thiogalactopyranoside. I use a 0.1 M solution. The formula weight is 238.3, so this is 0.238 g in 10 ml of water. Sterilize by filtration, then store in the freezer.

Xgal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside. I use a 20 mg/ml solution. It must be dissolved in DMSO (dimethyl sulfoxide) or dimethyl formamide, not water! It must be wrapped in foil to protect it from the light, and stored in the freezer.

5. After about 16 hours incubation at 37C, pick white colonies with a toothpick to 3 ml LB media containing 50 µg/ml ampicillin. Grow with shaking at >250 rpm overnight.

6. Prepare DNA for sequencing. Quality is critical! For automated sequencing, our best results are obtained using the Qiaprep spin miniprep kit (Qiagen). Quantitate the DNA concentration as described above.

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For pGEMTEZ, we normally sequence with T7 or SP6. Most facilities (including UCR's) will add such common primers at no charge.