Preparation of templates for DNA sequencing using the polymerase chain reaction
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Very often, molecular biologists will isolate a cDNA clone for a gene of interest. The sequence of the cDNA then needs to be determined to find out what protein is encoded. In this laboratory exercise you will have four cDNA clones that you need to sequence. Each of these cDNAs has already been placed into the multiple cloning site of the pCRII vector (see Figure 1). During the next three weeks, you will determine the sequence of the cDNA insert in each of these plasmids. Getting good quality DNA sequence data (reliable determination of all the bases without ambiguities) requires the use of a pure DNA template. Your mission for this week is to prepare a high quality template that you can later use for sequencing. The preparation of the sequencing template will be carried out using the polymerase chain reaction (PCR).

PCR is a very versatile tool for molecular biologists. This technique is sensitive enough to generate quite large amounts of DNA product beginning with very small amounts of starting material. The high sensitivity of PCR, however, means that one must be very careful. It is very important to avoid contamination of the samples with other plasmids or with PCR product from a previous reaction as the presence of only a few molecules of these could result in the production of unwanted PCR products. It is also critical to prevent any cross contamination between the different samples being analyzed.

You will start with a small amount of a crude plasmid preparation. While it is possible to use circular plasmid DNA as a template for DNA sequencing, you do not have as much of the plasmid as you would need and it is not sufficiently pure to serve as a good sequencing template. You will amplify the insert portion of the plasmid and purify the amplified insert for use as a sequencing template.

STOCK SOLUTIONS

- dNTP mix (2 mM dATP, 2 mM dTTP, 2 mM dCTP, 2 mM dGTP)
- -20 primer (2 mM) sequence = GTAAAACGACGGCCAGT
- R primer (2 mM) sequence = GGAAACAGCTATGACCATG
- 10X PCR Buffer II
- 25 mM MgCl₂
- Taq DNA polymerase (2 unit/mL)

PROCEDURE

1. Have ready 4 crude plasmid samples:

2. Prepare the 4 PCR reactions in thin-walled PCR tubes. Each reaction should contain a total volume of 100 µL. The final concentration of each component should be as follows:

- 1X PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl)
- 200 mM dATP
- 200 mM dTTP
- 200 mM dCTP
- 200 mM dGTP
- 200 nM -20 primer
- 200 nM R primer
- 1.0 mM MgCl₂
- 2 unit Taq DNA polymerase/reaction
- each tube should contain 1 µL of the template DNA
For greater repeatability it is best to prepare a master mix of all components except DNA and then aliquot the proper amount of this mix into each tube. The best way to do this is to first calculate the amount of each reagent needed per reaction, then add 5 times this amount into a master tube. Once the master mix is prepared you can distribute the proper amount of it to each individual reaction tube.

Mix your reagents together on ice and keep them on ice until you place them into the thermal cycler. Seal the tops of the tubes tightly.

3. Preheat the thermal cycler to 96°. Quickly transfer the tubes from the ice to the 96° heat block. Amplify the DNA as follows:

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\begin{align*}
3:00 & \rightarrow 96° \\
0:45 & \rightarrow 50° \\
1:00 & \rightarrow 72° \\
1:00 & \rightarrow 72° \\
6:00 & \rightarrow 96° \\
4° & \rightarrow 96° \\
\text{hold} & \rightarrow \\
\end{align*}
\]

35 cycles

note: this PCR reaction will take about 2:45 to complete.

4. Transfer each PCR product to a 500 μL tube. Precipitate the PCR product DNA by adding 5 μL 10 M ammonium acetate (mix well before adding ethanol) and 210 μL 100% ethanol to each tube. Mix well and place the tubes at -20° for 30 minutes. Centrifuge at 14,000 rpm at 4° for 30 minutes. Carefully remove all the liquid from the tube without disturbing the pellet. Allow the pellet to dry and add 15 μL water. After the DNA is dissolved in the water, store it at -20° for future use.
Figure 1. Plasmid vector pCRII with and without cDNA inserts. Not all restriction enzyme recognition sites are shown.