Cloning CYP1A partial cDNAs via RT-PCR

Cytochrome P4501A (CYP1A) is a liver microsomal enzyme. Its expression is induced at the transcriptional level by exposure to numerous environmental contaminants. Halogenated aromatic hydrocarbons, including dioxins and polychlorinated biphenyls (PCBs), are among the most potent CYP1A inducers. Other nonhalogenated aromatic hydrocarbons, such as those found in petroleum products and byproducts, also induce CYP1A expression. Thus, expression of CYP1A is an important biomarker of pollution exposure.

Step 1: Exposure of animals to contaminants. Fish were exposed to 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) via waterborne exposure or injection into the body cavity. After 24 hours, fish were sacrificed and dissected. Dr. Powell did this step.

Step 2: Total RNA isolation. Total RNA will be extracted from fish tissues using RNA STAT-60. The integrity of the RNA was assessed by examining it on an agarose/formaldehyde gel. *The presence of the two ribosomal RNA bands indicates intact RNA*.

Step 3: Total cDNA synthesis. The total RNA will be used as a template for reverse transcriptase (RT), an enzyme that catalyzes the synthesis of complementary DNA (cDNA) from RNA templates. The RT reaction will be primed using random hexamers.

Step 4: PCR with degenerate primers. cDNA synthesized in the RT reaction will be used as a template for the polymerase chain reaction (PCR). You will use PCR primers designed based on regions of conserved amino acids identified in the alignment of CYP1A protein sequences from a number of species. These primers must be degenerate, i.e. contain multiple nucleotide possibilities at positions where an amino acid was encoded by more than one codon. At positions where 3 or 4 nucleotides are possible, Inosine can be used, since it can form base pairs with A, C, G, or T.

Step 5: Analysis and Gel purification of the PCR products. *We will identify PCR products suitable for further study based on their size*. Proper size is predicted based on the positions of primer annealing in the aligned sequence and the approximate number of amino acids between the primers. We will cut the proper band from an agarose gel and purify it using the GeneClean II kit. This process isolates the band from other, non-specific PCR products and from the reagents in the PCR reaction.

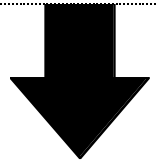
Step 6: Ligation of purified RT-PCR product into plasmid vector. Next we will mix the purified RT-PCR product with a linear plasmid vector, pGEMT-Easy. T4 DNA Ligase enzyme will catalye the formation of phosphodiester bonds between the fragment and vector DNAs. What features of each molecule facilitates their interaction?

Step 7: Transformation of *E. coli* with our newly-ligated plasmids. We will next take the ligation reactions and mix them with competent *E. coli* cells (JM109 strain). Competent cells have been treated with $CaCl_2$, which enables them to adsorb plasmid DNAs, facilitating their uptake into the cells interior. After the mixing and incubation procedures, the cells are spread onto LB-ampicillin plates and grown overnight.

Cells that take up a plasmid survive to divide and form colonies. Cells without plasmid die. *What is the basis of the selection*?

Most plasmids contain the insert sequence. A few may not, however. *How do we tell the difference simply by looking at the colonies and not the actual plasmid DNA?*

***QUESTION:* Why do we bother cloning our PCR products into a plasmid vector? What features of the plasmid make it advantageous?**



Step 8: Plasmid DNA minipreps. Next, we need to look at the recombinant plasmids transformed into *E. coli*. To have a look, we will pick several colonies from the plates and grow these cells in a liquid culture. We then harvest the cells by centrifugation and isolate the plasmid DNA using QIAprep columns. After recovering the plasmids, *how can we know they are constructed the way we thought they would be?* Hint: The sizes of DNA molecules will be an important source of information for us before. *How do we assess the size of the plasmid?*

Step 9: DNA sequence analysis. The size of a DNA fragment can be crucial to understanding its nature and relevance. However, there is no substitute for have the actual, detailed sequence information. Miniprep DNAs identified as good prospects will be sent to the DNA Sequencing Facility at the University of Maine (*Motto: We sequence wicked good!*). What will the sequence of our RT-PCR products tell us? How will we determine if it indeed encodes CYP1A?

Cloning partial cDNAs via RT-PCR: Process Overview