Illinois Institute of Technology
Biology 446-01

Laboratory Exercise 1: Polymerase Chain Reaction

Objective: To gain experience in running PCR and to learn to optimize a PCR reaction.

Safety first: We will use ethidium bromide to stain the DNA bands in agarose gels. Handle with care!

Background

The polymerase chain reaction, which won the 1991 Nobel prize in chemistry for Keri Mullis, is a technique by which a very small number of molecules, in some cases a single molecule, can be detected. The basis of the procedure relies on the same structural features of DNA that make it suitable for encoding genetic information in living cells: its duplex structure in which information is redundantly encodes on two complimentary strands. Each stand encodes all the information necessary to produce the entire molecule, and by splitting a part the two strands, each maybe used as a template to reproduce the original molecule thus creating two copies of the original molecule.

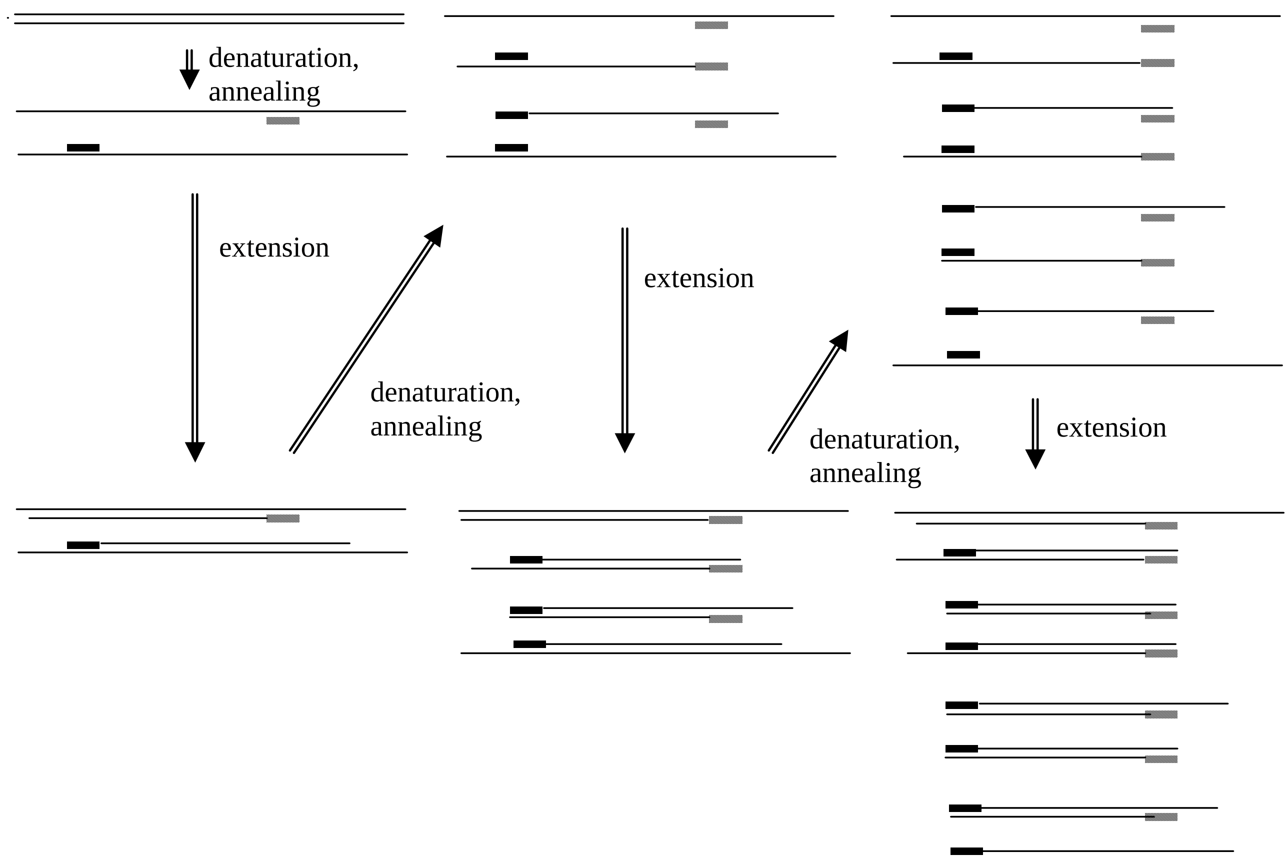
The specificity of the reaction is dictated by another salient feature of DNA. DNA is constructed of four nucleotides (adenine, cytosine, guanine, thymidine; or A, C, G, T), with the pairing between the two strands of the duplex strands following strict rules: A on one strand is invariable paired with T in the other, and C with G. This pairing is essential for the proper assembly of the two strands. We may thus interrogate a DNA sample for the presence of a specific sequence by constructing a synthetic DNA molecule, or primer, of complimentary sequence (i.e. A where ever we expect a T in the target sequence, etc.). This primer should only form a duplex structure a DNA sample at the location of the target sequence.

In practice, varying degrees of mismatch can be tolerated at low temperature, but as we raise the temperature, these less stable, imperfectly matched complexes are disrupted. At a critical temperature, only an exact match is tolerated. This annealing temperature is the single most critical parameter of the PCR reaction. It the annealing temperature is too low, alternative, imperfect sites create excess of false positive products. It the annealing temperature is too high, even the association with the fully complimentary target sequence is disrupted, preventing any reaction at all.

Since DNA is composed of 4 bases, there are four possible length one sequences: A, C, G and T. There are 4^2=16 possible length 2 sequences: AA, AC, AG, AT, CA, CC, CG, CT GA, GC, GG, GT, TA, TC, TG and TT. In general there are 4^n possible length n sequences.

This relationship can be turned around: in any random arrangement of DNA, we need to examine, on average 4 bases to match and arbitrary length 1 sequence, 16 bases to match a length 2 sequence, and 4^n bases to match a length n sequence. Since the human genome has ~3 000 000 000 ~ 4^{15} bases, a specific length 15 sequence can uniquely identify a specific location within the genome. In practice due to statistical factors, as well as non-random structures within the genome, primers slightly longer are often used. The primers we will be using in this experiment are 23 bases long.

In order to amplify the DNA, an enzyme, DNA polymerase, is used to add nucleotides to the ends of the primers in these small duplex structures. This enzyme adds specific bases such that the new strand is zippered up on the original sample, or template, strand to restore full complementarity and create a copy of the original DNA sample. Since the genome is so large and complex, in the limited time we have for the reaction, the polymerase cannot proceed to copy it in its entirely. However, this is used to our advantage. By using two primers, one targeted toward a region on each of the two strands of the sample such that the newly synthesized stands of each proceed toward the target region of the other. We can amplify only the region spanning these two target sites (See the figure below).



As indicated by the figure, the PCR reaction proceeds in three steps:

1. A denaturation step, in which the duplex nature of DNA is disrupted by high temperature, 94 °C.
2. An annealing step in which the synthetic primer DNA molecules are allowed to reform duplexes with the DNA sample, but only at the target sequence, as dictated by as carefully chosen annealing temperature.
3. An extension step, in which these duplexes are extended to reform the duplex structure disrupted in step 1.

These steps are repeated a number of times, sufficient to amplify the region between the two primers. Since each cycle of this duplication creates twice the number of molecules, the amplification proceeds exponentially, with 2^n molecules being produced after n steps. It is useful to recall that $2^{10} \sim 1000$, so after 20 steps we have approximately a million fold amplification, and after 35 amplification of $3x10^{10}$. This is enough to take a single molecule of DNA to an amount that is easily detectable with rudimentary apparatus.

The denaturing temperature, 94 or 95 °C, is high enough to denature long double-strand DNA. This is determined by the general property of DNA and is not a variable in a PCR reaction. For each cycle, 15-30 second is enough for the DNA to denature at high temperature, except for the first cycle, for which it takes longer to denature all the long DNA molecules.

For the extension step, the optimum temperature is between 72 and 74 °C for most DNA polymerases used in PCR reactions. The extension time depends on the length of the region of the DNA you want to amplify. The efficiency of the DNA polymerase is about 1000 base pair per minutes. Generally, not much can go wrong in this step, either.

However, one needs to use the right annealing temperature for each PCR reaction. If the annealing temperature is too high, the primers will not anneal to the template and not product will be made. It the annealing temperature is too low. Mismatched annealing can happen that results in none specific product. Approximately, the denaturing temperature (in °C) for a short double-strand DNA can be calculated as 2 times number of AT pairs plus 4 times the number of CG pairs. A good annealing temperature to try is 5-10 °C below the melting temperature the primers. Thus the two primer need to have similar melting temperature to guarantee good result.

In this lab exercise, you will carry out basic PCR experiments to amplify the coding sequences for the integrase and the `Excisionase from bacteriophage`. Both reactions will be set up the same way except the primers. We will, thus, set up a reaction mix without the primers and divide the mixture into aliquot and then add the primers:

Protocols:

To set up the PCR, follow these steps:

1. Set up the PCR mixtures in 200 µl microcentrifuge tubes by adding the following. You will need two PCR tubes. Please label them with your group number and the reaction name, e.g. name the reactions as Int, and Enase, respectively.

	Reaction*
Forward Primer **	15 µl
Reverse Primer	15 µl
Reaction mixture***	20

***The template for excisionase and integrase is bacterial lamda phage DNA digested with HindIII;**
****The primers will change according to the sequence you want to amplify:**

For excisionase:
(YZ644) Forward, ATGGGTTACTTGACACTTCAGGAGTGGAACGC
(YZ645) Reverse. GCTCATGACTTCGCCTTCTTCCC
For integrase:
(YZ646) Forward, GGTATGACGGGAAGAAGGCGAAGTCATGAGCG
(YZ647) Reverse, TTATTTGATTTCAATTTTGTCCCACTCCCTGC

All forward primers have tcgaaggagatctatacc in front of the primer and all reverse primers have tgggtctcgagggcccgc in front of the primer to facilitate the addition of the attachment site for in vitro recombination in a second PCR (see below). For example, the following are the actual primers for excicsionase:

YZ644=forward, tcgaaggagatctataccatgggttacttgacacttcaggagtggaacgc
YZ645=reverse, tgggtctcgagggcccgcgctcatgacttcgccttcttccc
The name of the primers are YZ644 and YZ 645, respectively, when we had the primers synthesized by a outside company.

*** the reaction mixture will be made up by the TAs for the whole class to avoid contamination. 120 μl of the mixture would contain:

Reagent	Volume	Final Concentration
H2O	50 μl	
25 mM $MgSO_4$	12 μl	2 mM MgCl2
10X PCR buffer (without Mg)	30 μl	1X PCR buffer
10X dNTP's	18 μl	200 μM each
Template	7 μl	
Taq NDA polymerase****	3 μl	

****NOTE: most polymerases from different company come with their own protocols. Many of them instruct you to add the polymerase last. If a PCR does not work you should make sure you add the enzyme last. In our case, it should not matter.**

2. Set up the PCR reaction with the following steps with hot lid:

   Step 1: 2 minutes at 95 °C (denature everything before the PCR cycle)
   Step 2: 30 seconds at 95 °C (denature)
   Step 3: 30 seconds at 60 °C (anneal)
   Step 4: 30 seconds at 72 °C (extension)
   Step 5: repeat steps 2 - 4 29 more times (cycle)
   Step 6: 2 minutes at 72 °C (final extension)
   Step 6: hold at 4 °C

   When the PCR is completed, the TAs will run a gel of your PCR and purify the products which will be used in a second PCR with the following primers:
   forward ccaatccccacaagtttgtacaaaaaagcaggctcgaaggagatctatacc
   reverse ggaatggggggaccactttgtacaagaaagctgggtctcgagggcccgc to facilitate in vitro recombination.

The PCR tubes of the second PCR will be removed and stored at 4 °C for your use in the next lab class.

We can carry out the PCR in one step if we synthesize long primers instead of shorter overlapping primers. For example, the two-step PCR for excicsionase is equivalent of using the following primers with one PCR reaction:

forward, ccaatccccacaagtttgtacaaaaaagcaggctcgaaggagatctataccatgggttacttgacacttcaggagtggaacgc

reverse, ggaatgggggaccactttgtacaagaaagctggtgggtctcgagggcccgcgctcatgacttcgccttcttccc

The reason that we are going to use shorter primers is cost effectiveness. Remember that the primers for the second PCR only need to be synthesized once and they can be used in the second PCR for cloning any gene, if the primers in the first PCR have the blue bases.

Lab day 2

Agerose gel:

1. Prepare 60 ml of a 1% agarose solution (i.e. 0.6 g agarose, 60 ml TBE with ethidium bromide). Melt the agarose using a microwave. Be careful not to allow the solution to boil over. Watch the solution and stop the microwave as soon as it starts to boil. Use a paper towel to hold the hot flask and take it out of the microwave. Slowly swirl the solution so that it will not burst out. Pour the solution into the apparatus tray. Be sure to insert the gel combs immediately, before it hardens.

   Once the gel has hardened, remove the comb, and place the apparatus in such an orientation that the combs are close to the negative electrode. Fill the apparatus with the TBE running buffer (which also contains the same concentration of ethidium bromide) until the level just rises above the top of the gel and fills the wells.

2. Thaw your samples and add 4 µl of the gel-loading dye to each sample.
3. Load your samples into the assigned wells of the agarose gel. Be very careful in loading your samples so that you do not spill-over into adjacent lanes!
4. As the agarose gel contains ethidium bromide, it may be viewed directly under UV light following the completion of the run. We are using a Gel documenter to take images of the gels.
5. Using a UV light box with a UV protection cover, cut the PCR products from the gel and purify them using the gel purification kit from Qiagen following the manufacturer's protocol.
   Re member do NOT look directly to the gel when the UV is on in any situation.

6. Set up a BP in vitro recombination reaction following the manufacturer's protocols.