

Lab Worksheets

Investigating the Molecular Mechanism of Evolution: Mutation and Natural Selection

Tina Hubler, Patti Adams and Jonathan Scammell

<u>Worksheet</u>	<u>Activity</u>
1	Preparation
2	Activity I
3	Activity II
4	Activity III

Lab Worksheet 1 Preparation

Lab 1 PCR and Electrophoresis

PCR background

PCR is a powerful tool that allows researchers to produce millions of copies of selected regions of DNA. This quantity of DNA is required for downstream applications such as DNA fingerprinting and DNA sequencing. The *in vitro* copying of DNA in the laboratory follows the same basic steps that occur *in vivo* in the cell each time DNA is replicated prior to cell division. However there are some important differences. First, denaturing (separating) DNA is accomplished by heating the sample rather than by enzymatic activity (helicase), as it is accomplished in the cell. Second DNA primers are used to initiate new DNA synthesis, rather than RNA primers that are synthesized by primase in cells. Third, Okazaki fragments are not created because the DNA is completely denatured by heat. Fourth, as a consequence of these differences between *in vitro* and *in vivo* DNA synthesis, a thermostable DNA polymerase is the only enzyme required for synthesis of new DNA strands. The most commonly used thermostable polymerase originated from the thermophilic bacterium *Thermus aquaticus* and is abbreviated as *Taq* DNA polymerase.

The reagents needed for PCR are: (1) a template, the DNA to be copied, (2) nucleotide triphosphates, the nucleotide subunits of DNA, (3) thermostable DNA polymerase, (4) DNA primers complementary to the ends of the DNA region to be copied, (5) buffer, to maintain pH and ionic strength of the reaction mixture and (6) nuclease-free water. Supplies and equipment needed for PCR are: (1) a PCR cycler with a programmable heat block that allows one to change the temperature of the reaction mixture, (2) PCR tubes to hold the reaction mixture, and (3) pipettors and sterile pipet tips.

After the reagents are added to the PCR tube, reaction mixtures are placed into a PCR cycler and the stages of PCR are initiated. The stages are: (1) denaturation at 92-95 °C, (2) annealing of primers to the template DNA at approximately 45-65 °C and (3) synthesis of new DNA at approximately 68-72 °C. Denaturation separates the DNA strands so that each may be used as a template for new DNA synthesis. Annealing requires lower temperatures to allow nucleic acids to associate by complementary base pairing. The primers are usually 15-25 nucleotides in length and are complementary to the ends of the region of DNA to be copied. The primer length and composition (percentage of A, T, C, and G) largely determine the annealing temperature. For example, longer primers with higher percentages of C and G are used with higher annealing temperatures. Synthesis of DNA requires the enzyme DNA Polymerase. As is the case for all enzymes, its optimal function depends on pH, ionic strength and temperature. The manufacturer of the polymerase will recommend the optimal conditions and the time needed for the synthesis.

The above three steps represent one PCR cycle that produces two DNA molecules from each template molecule. In the next cycle, the two DNA molecules serve as template and four DNA molecules are produced. The amount of DNA doubles with each cycle (i.e. 1, 2, 4, 8, 16, 32, etc.). This exponential amplification can produce more than 1 million copies of DNA after 20 to 30 cycles.

A typical PCR setup and conditions for a 1 kb template are listed below:

Setup:

10X reaction buffer (with MgCl ₂)	5 ul
dNTP mix (10 mM of each dNTP)	1 ul

Taq DNA Polymerase (5 U/ul) 0.25ul
downstream primer (50 pmol/ul) 1 ul
upstream primer (50 pmol/ul) 1 ul
template DNA (10-100 ng/ul) 1-2 ul
nuclease-free water (adjust to a final 50 ul)

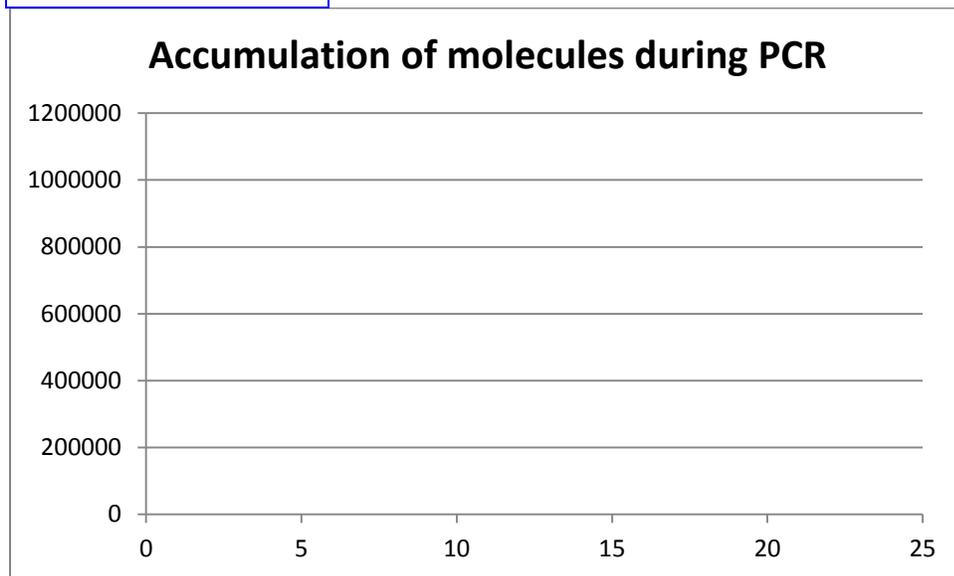
Cycle conditions:

95 °C 5 min initial denature step
25-35 cycles:
95 °C 30 sec denature step
55 °C - 65 °C 30 - 60 sec annealing primer
72 °C 90 - 120 sec extension step
72 °C 7 min final extension step

PCR student activity

As an introductory activity, prepare a graphical representation of the number of DNA molecules that would accumulate during PCR cycling if you start with 1 molecule. Plot the number of cycles on the x-axis and the number of molecules on the y-axis.

Number of cycles	Number of molecules
1	2
2	4
3	8
5	32
10	1024
15	32768
20	1048576



Electrophoresis background

Electrophoresis is a common technique used in molecular laboratories to separate mixtures of charged molecules. In our activities the molecules are separated according to size. An agarose gel may be used as the supporting medium to separate nucleic acids. The gels are made by dissolving solid agarose in boiling buffer, pouring the partially cooled solution into a casting tray, and allowing it to solidify by cooling to room temperature. The gel casting tray includes a comb that forms sample wells as the gel solidifies. Agarose gels are a porous matrix, with the pore size depending on the concentration of agarose used. Higher concentrations of agarose yield gels with smaller pores. 1.2 - 1.5 % agarose gels are used to separate DNA molecules from 200 to 10000 base pairs in size.

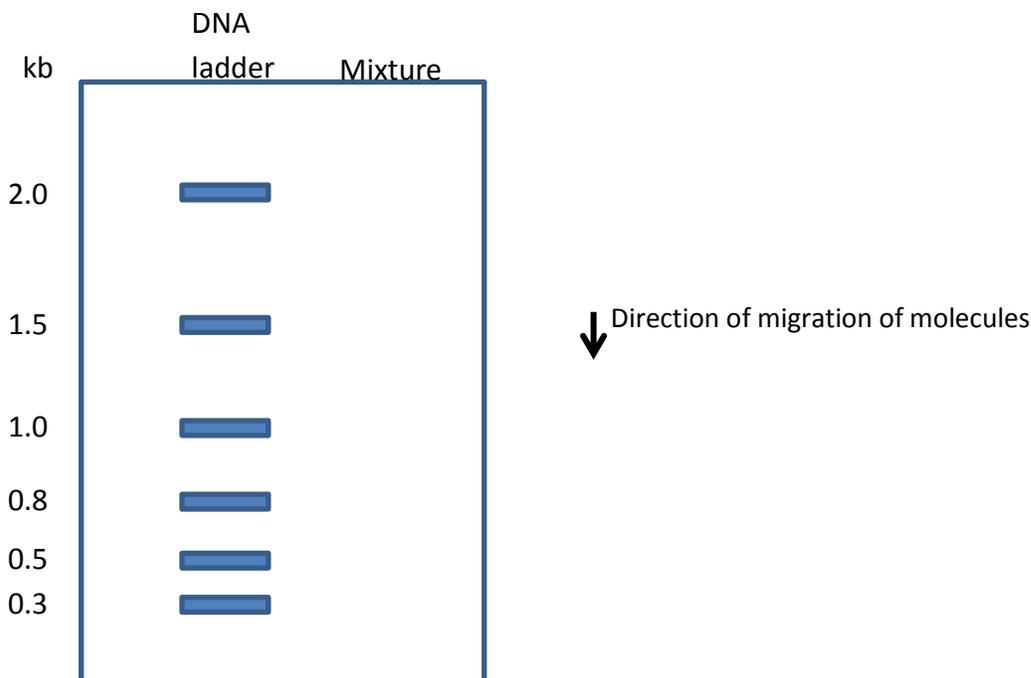
The gel is submerged in an electrophoretic chamber containing buffer (usually Tris Acetate-EDTA, pH=8). Samples are loaded into the sample wells and current is applied to the chamber. At pH=8, nucleic acids are negatively charged and travel under the influence of the electric field toward the opposite end of the gel (the positive electrode). Smaller molecules travel through the pores more quickly and migrate further in the gel.

Electrophoresis buffer is used to control pH and ionic strength of the gel and chamber. In contrast, sample buffer containing 10-20% glycerol is sometimes added to samples to cause samples to settle to the bottom of the sample well before electrophoresis begins.

After electrophoresis, sample components are usually not visible unless stained. Agarose gels are commonly stained with either methylene blue or ethidium bromide. Both stains have affinity for nucleic acids. Ethidium bromide is more sensitive and allows one to visualize smaller quantities of DNA. However, it is a mutagen and requires ultraviolet light for visualization. If sufficient quantities of nucleic acids are present, methylene blue is a safer alternative for visualization.

Electrophoresis student activity

Predict the migration of molecules from a mixture of DNA. Assume a DNA ladder is added to one well in a gel and the following mixture of molecules is added to the second well: 1500 bp, 1000 bp, and 500 bp of DNA. Predict how far the individual molecules from the mixture will migrate and draw the positions on the gel below.



Lab Worksheet 2 Activity I

Lab 2 PCR of Nonfunctional DNA Sequences

This activity will help students understand that lack of selective pressure on nonfunctional sequences results in sequence variation, even in closely related organisms. We will use PCR to show that a nonfunctional region exhibits variability in two species of New World primates (NWP), squirrel monkeys and owl monkeys. DNA sequences for the variable region will be used to identify the insertion of an Alu element (a type of mobile genetic sequence).

Plasmid DNA containing the variable region from owl monkeys or squirrel monkeys is used as the template for PCR. The components needed for a 25 μ L reaction in a 200 μ L PCR tube and the PCR conditions are listed below. Following PCR, samples are loaded onto 1% agarose gels and electrophoresed at 100 V for 55-60 minutes. A DNA ladder may be loaded into a separate well to estimate PCR product size. The gels are stained with methylene blue gel stain according to the manufacturer's instructions. PCR products are visualized by placing the gel on a white illuminated surface.

PCR set up

PCR Components	
Component	Volume
DNA template (dilute to 10 ng/ μ L)	1.0 μ L
Forward primer (10 μ M) 5'-AGTTCCTCTCTACCTTGTACC-3'	1.0 μ L
Reverse primer (10 μ M) 5'-GCCCTACTCTTGCAATTAATGC-3'	1.0 μ L
GoTaq [®] Green Master Mix	12.5 μ L
Nuclease-free water	9.5 μ L

PCR Conditions	
Stage	Conditions
Initial denaturation	94 °C, 1 minute
Cycling (30 times):	
Denaturation	94 °C, 1 minute
Annealing	54 °C, 30 seconds
Elongation	72 °C, 90 seconds
Final extension	72 °C, 5 minutes

Observations

Sample PCR product size

Owl monkey

Squirrel monkey

Investigating the reason for the difference in size of the PCR products

The DNA sequence for the squirrel monkey PCR product will be provided to students electronically. The sequence that contributes to the difference in size of the squirrel monkey region is an Alu element, a type of mobile DNA sequence that has been inserted into this region. Students can determine that an insertion is present by accessing the BLAST page (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and following these steps. Select “Align”, under Specialized BLAST. Copy and paste the squirrel monkey sequence into the query box and the owl monkey sequence into the subject box. Select “somewhat similar sequences” under Program Selection. At the bottom of the page select “BLAST.” Students can identify the insertion as an Alu element by following these steps at the BLAST page. Select “nucleotide blast.” Copy and paste the squirrel monkey sequence into the

query box. In the second box, select the database "Human Alu repeat elements" from the dropdown list. At the bottom of the page select "BLAST."

Observations

Approximately how many nucleotides are inserted into the squirrel monkey region?

Toward which end (5' or 3') of the PCR sequence does the Alu sequence occur?

Conclusions

Owl monkeys and squirrel monkeys are closely related primate species. What does your observation of PCR products tell you about DNA sequences in nonfunctional regions?

In this example what is the form of genetic variability that occurs in a nonfunctional region of the genome?

Based on lecture discussions of mutation and natural selection, describe in your own words why mutations such as insertions may persist in nonfunctional regions throughout generations of organisms and lead to high variability in these regions.

Lab Worksheet 3 Activity II

This activity will help students visualize conservation of functional DNA sequences. We will demonstrate by PCR that the DNA sequence of an important gene is conserved among two groups of primates. Old World primates (OWP), such as rhesus monkeys, chimpanzees and humans are found primarily in Africa and southern Asia. In contrast, New World primates (NWP) such as squirrel monkeys, marmosets and owl monkeys inhabit South America. OWP and NWP are thought to have evolved independently from a common prosimian ancestor over 30 million years ago. Still, functional DNA sequences have been conserved. The sequences of the PCR products will be provided to students electronically to enable students to evaluate the similarity in the DNA sequences.

In this activity, students will compare the size and DNA sequence of the primate chorionic gonadotropin (CG) gene. CG is a peptide hormone made in primates by placental cells during the first ten weeks of pregnancy. It supports the implantation and development of the fetus. CG therefore represents a critical gene for reproductive success in primates.

Plasmid DNA containing the CG gene from humans, owl monkeys or squirrel monkeys will be used as the template for PCR. The PCR components and settings are indicated below. PCR products are fractionated and visualized using methylene blue stain.

PCR set up

PCR Components	
Component	Volume
DNA template (dilute to 10 ng/ μ L)	1.0 μ L
Forward primer (10 μ M) 5'-GCACCAAGGATGGAGATG-3'	1.0 μ L
Reverse primer (10 μ M) 5'-GCGGATTGAGAAGCCTTA-3'	1.0 μ L
GoTaq [®] Green Master Mix	12.5 μ L
Nuclease-free water	9.5 μ L

PCR Conditions	
Stage	Conditions
Initial denaturation	94 °C, 1 minute
Cycling (30 times):	
Denaturation	94 °C, 1 minute
Annealing	54 °C, 30 seconds
Elongation	72 °C, 90 seconds
Final extension	72 °C, 5 minutes

Observations

Sample PCR product size

Owl monkey

Squirrel monkey

Human

Determining the similarity between OWP and NWP CG gene sequences

DNA sequences for the PCR products and for the unrelated genes for growth hormone and oxytocin will be used for sequence comparisons. On the BLAST web page (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) select “align two sequences.” Copy and paste the human sequence into the query box. Copy and paste the squirrel monkey sequence into the subject box. At the bottom of the page select “BLAST.” Results will list the % identical nucleotides. Repeat this to compare the other sequences.

Observations

Genes compared _____ % Identical Nucleotides

hCG vs. om CG

hCG vs. sm CG

sm vs. om CG

hCG vs. h growth hormone

h CG vs. h oxytocin

Conclusions

What do these results suggest about the similarity of functional DNA sequences?

Why do you think the sequence similarity is higher among NWP?

Why do you think 100% sequence similarity is not observed in the functional sequences?

Based on lecture discussions of mutation and natural selection, describe in your own words why conserved sequences may persist in functional regions of genomes.

Worksheet 4 Activity III

Question sheet for *Into the Jungle* Sean B. Carroll, *In Cold blood: the Tale of the Icefish*

A Bloodless Fish?

1. The bloodless fish was discovered accidentally during commercial whaling expeditions. One of the researchers, Rudd, noted "it was impossible because...all vertebrates share...presence of O₂-binding hemoglobin in red blood cells". What does this tell you about the nature of current knowledge (dogma)?
2. When blood was centrifuged what was absent in the bloodless icefish?
3. How does O₂ capacity of icefish compare to red-blooded relatives?

A Matter of Degrees

4. Arctic waters are on average -1.9 °C. The freezing point of water is 0 °C. Why don't icefish freeze? Remember freezing point depression from chemistry and what causes it.
5. What geologic changes may have occurred around Antarctica ~ 33-34 MYA (million years ago)?
6. What happened to water temperatures 20 million years later (~ 15 MYA), about the same time that icefish fossils appear in fossil records?
7. What type of molecule is AFGP? What does glyco mean?
8. The AFGP are composed of repeats of what 3 amino acids?
9. Draw the structure of threonine and show where you can add sugar groups to an amino acid.
10. How does AFGP prevent ice crystals forming in the icefish circulation?
11. The AFGP DNA in icefish is extraordinarily similar to the trypsinogen gene. What regions of the AFGP gene are found in the trypsinogen gene?

12. What does this degree of similarity suggest?
13. In what part of the trypsinogen gene is a repetitive sequence found?
14. How is this region (in #13) of a gene normally used?
15. The relationship of AFGP to trypsinogen suggests a mechanism for genetic (molecular) evolution. It is:
 - a) tinkering with materials (DNA) already available
 - b) inventing new DNA

Fossil Genes

16. Hemoglobin protein is made from α -globin and β -globin genes. What is the status of these in icefish?
 α -globin is _____ β -globin is _____
17. A nonfunctional remnant of a gene is a _____ (a molecular fossil).
18. What features of the icefish cardiovascular system allow the icefish to survive without hemoglobin?
 a) _____ b) _____ c) _____
19. What happened to the myoglobin gene in icefish that caused it to become a molecular fossil?

The DNA Record of Evolution

20. Paleontologist Simpson states "species evolve ...as if they are adapting as best they could to a changing world, and not at all as if they were moving toward a set goal".
 Below is a summary of the proposed evolution of icefish based on geological and molecular evidence. Fill in the blanks.

Time, MYA

geologic conditions

Antarctic fish types

> 40	warm water	red-blooded fish (sharks, rays, catfish)
33-34	antarctic separated	
15-25	water temperatures dropped to below freezing	fish with AFGPs (e.g. notothenoids)
14	thick sea ice formed	
2-8		icefish with loss of _____ gene function
<2 and still occurring		icefish with changes in _____ genes

21. Evolution is not necessarily progressive i.e. developing new traits. Sometimes genes and functions are _____.

22. What may happen to icefish if waters warm again?

_____ or _____

23. In your own words, explain how gene duplication and selection may have resulted in the evolution of icefish.

.