Purpose: To isolate and characterize DNA from liver (cow, pork, chicken) and amplify cytochrome-b gene.

A. Isolation of Nucleic Acids

1. Classical DNA and RNA Isolation and Purification. Nucleic acids are some of the most polar biopolymers and therefore are very soluble in polar solvents and are precipitated by non-polar solvents. In prokaryotes, DNA is double stranded and circular and is found in the cytoplasm. In eukaryotes DNA is located in the nucleus, mitochondria, and chloroplasts. DNA in the nucleus is double stranded linear whereas in the chloroplasts and mitochondria it is double stranded and circular. In prokaryotes DNA is relatively free of protein, but in the eukaryotic nucleus DNA is associated with basic proteins, histones.

Classical isolation and purification of DNA took 4 steps: (a) the disruption of cells and organelles to release DNA, (b) inactivation of enzymes that hydrolyze DNA, (c) dissociation and denaturation of protein, and (d) solvent extraction and precipitation of DNA. Cells were broken by grinding tissues or by treatment with lysozyme (bacteria). Chelating agents were added to remove metal ions required for nuclease activity. DNA-protein associations were disrupted by mixing the ground tissue with SDS, phenol and organic solvents. From these steps, DNA remains in the aqueous phase from which it could be easily precipitated with cold ethanol. DNA precipitate was then dissolved in buffer and re-treated with phenol or organic solvents to remove the last traces of protein. RNA could be removed by treatment with RNase (deoxyribonuclease free) or by alkalai treatment.

Classical RNA isolation began with solvent extraction that denatured proteins and inactivated RNases. The RNA containing aqueous phase is re-extracted with phenol-buffer. Messenger RNA in the aqueous phase was precipitated by 0.1M NaCl and 70% ethanol. Ribosomal RNA was precipitated with 3 M Na-acetate and 70% ethanol (in which the small t-RNA’s are soluble) and the t-RNA’s were precipitated in 1 M NaCl and cold 66% ethanol.

Both of these procedures required extraction relatively large volumes of tissue along with using caustic chemicals such as phenol. Further, phenol had to be re-distilled prior to use. Using spin columns DNA can be isolated from small tissue samples without using caustic chemicals.

2. Silica column DNA isolation. These procedures utilize small spin columns of silica. Tissue samples (about 25 mg) are mixed with enzymes (RNase and proteinase-K) to disrupt and digest tissue components except DNA. The disrupted cell mixture is then placed in a spin-column and then centrifuged. In the column DNA sticks to the silica whereas most other molecules are passed through. The column is then washed with ethanol and spun, then washed with water and spun. The last step is the elution of DNA from the column.

Using these procedures, 25 mg of liver can produce 25 µg of DNA. This is roughly 40 nmoles of DNA in a volume that is about 1 ml (assumes the average base-pair molecular weight
is 618 daltons). A pure preparation of DNA or RNA should have a ratio of Abs$_{260/280}$ of 1.8 to 2.0. Contamination with protein will lower this ratio. A good UV-spectrophotometer can read concentrations of DNA to less than 5 µg/ml. We will be using the Sigma-GenElute Mammalian Genomic DNA Miniprep kits to isolate DNA that we will examine UV spectra and use as a template in Lab 2 to amplify by polymerase-chain-reaction (PCR) a portion of the cytochrome-b gene.

B. Polymerase Chain Reaction (PCR).

DNA polymerases all require besides the substrate dNTP’s (dATP, dTTP, dGTP, dCTP), Mg$^{++}$, buffer and primers. Primers are chemically synthesized single stranded oligonucleotides usually 15 to 30 bases in length. DNA polymerase only works when it can attach the first nucleotide to an existing 3’-OH from double stranded DNA. (Note that RNA polymerase does not require primers). So, to begin a gene amplification, one needs to design primers so that they will anneal to the gene at particular spot. Two primers are used, the forward and reverse primer each of which subtends the region to be amplified.

PCR reactions rely upon the heat stability of DNA polymerases from extreme thermophilic bacteria. The classic DNA polymerase came from *Thermus aquaticus* a bacterium isolated from a hot-spring. *T. aquaticus* DNA polymerase is called Taq polymerase and is relatively stable to boiling! This is an important point for it allows reaction mixtures to be heated to 95°C to convert ds-DNA to ss-DNA without destroying Taq polymerase. DNA polymerase from most organisms (or any enzyme for that matter) would be totally destroyed by such heat, but not Taq polymerase.

PCR is done by mixing chromosomal or plasmid DNA with a master mix (containing all dNTP’s, Mg$^{++}$, primers) and initiated by adding Taq polymerase. The tubes are put into a thermal cycler (commonly called a PCR machine) which heats and cools the tubes following a thermal program for each cycle. For example, a cycle we will do will be:

1. heat tube to 95°C for 1 min = makes all DNA single stranded.
2. cool to 52°C for 1 min = allows primers to anneal to template DNA (chromosomal or plasmid DNA).
3. heat to 72°C for 1 min = the optimal temperature for Taq polymerase allowing DNA synthesis from the primers, this is called “extension”.

For each cycle there is a doubling of DNA corresponding to the region subtended by the primers. In a typical run, such as we will do, 30 cycles are performed resulting in an exponential amplification. If the tube had only one copy of the gene being amplified, after 30 cycles there would be 5 x 10$^8$ copies. This is amplification!

After amplification, the PCR products (amplicons) will be examined with and without restriction enzyme digestion and separated on agarose gel electrophoresis. In this electrophoresis, the bands are separated based on size and they will be visualized by staining the gel with ethidium bromide which is a DNA intercalator. Ethidium bromide is relatively non fluorescent by itself, but increases its fluorescence when intercalated into dsDNA. The gels will
be viewed with ultraviolet light to stimulate ethidium bromide fluorescence in DNA Lab 2.

C. Materials.

Liver tissue, fresh or frozen, kept on ice. From cow, pork or chicken.
Waterbath, 55°C.
Waterbath or Tempblock at 70°C.
Sterile yellow pipet tips.
1.5 ml sterile micro-centrifuge tubes.
Microcentrifuge.
Ethanol (Absolute).
Molecular Biology grade Reagent Water.
Sigma-GenElute kits.

D. Procedure - DNA isolation from liver.

Tissue Prep/Dissolution
1. Weigh a 1.5 ml centrifuge tube.
2. Cut a piece of liver to be close to 25 mg, insert in tube, weigh the tube again and record the
tissue weight, mash/mince with a plastic pestle.
3. Add 180 µl of Lysis Solution T and then 20 µl of Proteinase K solution (0.25ml water to stock
bottle).
4. Immediately mix by vortexing and place into the 55°C waterbath.
5. Check the tube every 5 min, and vortex. Continue the incubation until the tissue sample
becomes homogeneous. This could happen rapidly or take 2 hours.
6. Add 20 µl of RNase A solution, incubate 2 min at room temperature.
7. Add 200 µl of Lysis Solution C and vortex, place in the 70°C waterbath for 10 minutes.

Spin Column Prep
8. Spin column preparation: To a spin column add 500 µl of Column Preparation Solution,
centrifuge at 12,000 x g (approx 13 k rpm) for 1 minute.

DNA Separation
9. Add 200 µl of ethanol to the tissue lysate from step 7, vortex 10-20 sec to get it homogenous.
10. Transfer the contents of the tissue lysate to the spin-column tube...use a wide bore pipet to
avoid shearing DNA. Centrifuge at >6500 x g (approx 10 k rpm) for 1 minute. Discard
the tube.
11. Place the spin column in a fresh tube, add 500 µl of Wash Solution (previously prepared
with ethanol: add 10 ml ethanol to Wash Solution Concentrate bottle). Centrifuge at
>6,500 x g for 1 minute. Discard the collection tube, place spin column in a new tube.
12. Add another 500 µl of Wash Solution, centrifuge 3 minutes at maximum centrifuge speed.
This must free the column of ethanol. Do a second centrifugation if necessary.
13. Elute DNA by pipetting in 200 µl of Elution Solution directly into the center of the spin
column. Incubate 5 minutes at room temperature, then centrifuge at >6,500 x g for 1
minute. This should be short-term stored at 2-8°C or frozen for long term storage.

E. UV Spectrophotometry.
1. To a pre-warmed up UV-Vis spectrophotometer, match at 260nm two 1 ml cuvettes with TE buffer. (TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0-8.5).

2. To the sample cuvette add 10 µl of your DNA preparation. The absorbancy should be in the linear range of the instrument (0.03 to 0.7 Abs). For calculation, a 1.0 absorbancy at 260 nm of double stranded DNA corresponds to 50 µg DNA/ml.

3. If good measurable absorbancy is achieved, then scan the sample from 200 => 400 nm.

F. PCR Procedure.

1. Teaching assistants will prepare a master mix. The mix will contain:

   10X buffer  => 5 µl
   MgCl₂  => 5 µl
   dNTP (1 mM) => 10 µl
   Forward primer (1 µM) => 5 µl
   Reverse primer (1 µM) => 5 µl
   Taq polymerase => 0.125 µl or 5 units.µl.

   Each group will put 30 µl of master mix into a PCR tube.

2. Add your DNA sample in 20 µl. The amount of DNA you add is important, it should be in the about 100 ng. Calculate this from the UV data. If the DNA volume is less than 20 µl, make up the difference with reagent grade water.

3. PCR Program Steps:
   A. Heat to 95°C for 3 minutes (to get all the DNA single stranded, inactivate any enzymes or contaminants).
   B. 30 cycles of 95°C for 1 min => 52°C for 1 min => 72°C for 1 min.
   C. Last cycle keep at 72°C for 10 min to finish partial copies.
   D. Chill to 4°C until tubes are removed from the machine (next day).

4. Because the amplification will take a long time, the Teaching Assistants will remove the tubes and keep them chilled until next lab.

The database you can find out about cytochrome-b is found at [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov). You need to get the sequences for your species of liver. *Sus scrofa* is the scientific name for the common pig. *Bos taurus* is the name for cattle and *Gallus gallus* is the name for the common chicken. **Bring a print-out of the cytochrome-b sequence to the next lab for the species you used in DNA Lab 1.**