This Week:

PCR amplification of the ribosomal locus from Your gDNA preps

Polymerase Chain Reaction (PCR)
- Used to amplify DNA exponentially.
- Sequences for amplification can be chosen precisely
- A cornerstone of molecular biology

Why do PCR?
- Amplify a specific DNA sequence for use in cloning
- Generate enough starting template for sequencing
- Amplify a scant signal sufficiently to detect it

Polymerase Chain Reaction (PCR)
For every single template of double-stranded DNA...

...a cycle of PCR creates two products.

PCR: Reaction components

1. A thermostable DNA polymerase
- Catalyzes template-dependent synthesis of DNA.
- Polymerization rate of Taq is ~ 2000 bp/minute

II. A pair of synthetic oligonucleotides to prime DNA synthesis
- Carefully designed primers are crucial to success; pay attention to thermodynamic and structural principles (more about this later in the class)

III. Deoxynucleoside triphosphates (dNTPs)
- 200-250 μM of each dNTP in equimolar amounts

IV. Divalent cations (usually Mg++)
- DNA polymerase requires divalent cation for activity.
- dNTPs and oligonucleotides can sequester Mg++, so concentration of cation should exceed molar concentration of phosphate groups contributed by dNTPs plus primers. 1.5mM Mg++ is common, but may need to play with this

V. Buffer: 10mM Tris-CI buffer to maintain pH; monovalent cations (usually 50mM KCl)

VI. Template DNA
- Can be single- or double-stranded
- Technically only need one DNA molecule for amplification

In the Tue/Wed lab session, all these components except template will be pre-mixed in your “master mix”.

Template = the DNA from food samples – not very abundant after being processed
**PCR: the cycling reactions**

The essence of PCR is temperature cycling.

Why?

**PCR: Overview of the cycling reactions**

Step 1. Initial denaturation
94°C for 1-4 minutes to dissociate all double-stranded template.

Step 2. Cycling
   1. Denaturation step = 94°C.
   2. Primer annealing = Primer $T_m$ (or slightly cooler ~ 5°C).
   3. Elongation = Taq polymerase optimal activity 72°C

Step 3. Final elongation of partially elongated fragments (72°C for 10 min)
Step 4. Hold reaction mix @ 4°C (e.g. until researcher gets back from lunch).

**PCR: the cycling reactions**

I. Denaturation at 94°C:
   - double stranded DNA melts open to single stranded DNA
   - all enzymatic reactions stop (for example: the extension from a previous cycle).

II. Annealing at 60°C:
   The primers are floating around (Brownian motion).
   Random, transient hydrogen bonds form and break between the primer (single stranded) and the template (still single-stranded from the denaturation step).
   When primers exactly match template sequence, the bonds are stronger and the resultant duplex (template and primer) lasts long enough for DNA polymerase to attach and start copying the template.
   Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.
   Short sequences anneal at this temperature, but not entire template.
   Rule of thumb: annealing temperature = primer $T_m$ minus 5-10°C

III. Extension at 72°C:
   68 - 72°C is the ideal working temperature for most commercially available polymerases.
   Perfectly complementary DNA duplexes are strong enough to persist at this higher temperature. Most incorrectly matched duplexes will disengage, eliminating extension of the corresponding incorrect fragments.
   One by one, nucleoside triphosphates (complementary to the template) are added to the primer on the 3' end.
   Rule of thumb: extension time should be ~ 1 minute for every thousand bp of product.
expandable

PCR: the cycling reactions

III. Extension at 72°C

Polymerase Chain Reaction

In the first cycle, extension from one primer proceeds beyond the sequence complementary to the binding site of the other primer.

In the 2nd cycle, the first molecules are produced whose length is equal to the segment of DNA limited by the binding sites of the primers.

After the 3rd cycle, this discrete segment of DNA is amplified geometrically, whereas the longer (not end-limited) amplification products accumulate arithmetically.

Polymerase Chain Reaction: History

First proposed in 1971 (Khorana and colleagues):
DNA polymerases synthesize DNA from nucleoside triphosphates, producing sequence complementary to a single-stranded template.
DNA polymerase could be used to copy a strand of DNA in vitro!

But not feasible:
- gene sequencing was not yet an option (DNA polymerase)
- no thermostable polymerase, so have to add new each cycle
- synthesis of oligonucleotide primers was not “easy” yet

Polymerase Chain Reaction: History

Idea re-born in 1986 (K. Mullis)
- DNA polymerase I from E. coli for amplification of DNA sequence
- But, problems:
  i) Denaturation steps inactivated DNA polymerase I. Researchers had to manually add enzyme to each cycle.
  ii) Researchers had to manually move tubes between three waterbaths at different temperatures

Polymerase Chain Reaction: History

- Modified two years later to use thermostable DNA polymerase from T. aquaticus (a bacterium isolated by Thomas Brock from a hot spring in Yellowstone Nat’l. Park)
  - The maximal catalytic activity of Taq polymerase is 75-70°C, and its half-life at 94°C (melting temperature) is 1.6 hours.

Polymerase Chain Reaction: History

- Soon afterward followed the development of automated thermal cyclers, which can heat and cool the tubes with the reaction mixture in a very short time.
  No more manual thermal cycling!
Polymerase Chain Reaction: History

- Mullis received a $10,000 bonus from his employer, Cetus, for his idea.
- Cetus later sold the patent to La Roche for $300,000,000.
- In 1993, Mullis received the Nobel Prize in Chemistry.

Food for thought:
...there are people who think that cells near deep hydrothermal vents actually conducted the first PCR, perhaps facilitating the first reproduction of biological information.

Common transgenes:

- Bacillus thuringiensis (Bt) endotoxin Cry1Ac; Cry1Ab; Cry3A; Cry1F: combats insect larvae that bore into plants
- Glyphosate resistance gene (Roundup-Ready): Modification of 5-enolpyruvylshikimate-3-phosphate synthase; allows application of pesticides early and efficiently
- PRSV coat protein: Papaya ringspot virus coat protein – confers resistance to widespread virus affecting papaya
- Other virus coat proteins: Cucumber mosaic virus, zucchini yellow mosaic virus, watermelon mosaic virus, engineered into squash
- Potato virus Y coat protein (potatoes)
- Resistance to the herbicide glufosinate (and others)... mostly canola, corn and cotton

The history of Bt as an organic pesticide in agriculture

*Bacillus thuringiensis* (Bt) is a Gram-positive, spore-forming bacterium found almost everywhere in the world. Surveys have indicated that Bt is distributed in the soil sparsely but frequently worldwide. Although it can live in the soil, on the phylloplane (leaf surface) and elsewhere, it is frequently found in insect “cadavers.”
First isolated *Bacillus thuringiensis* (Bt) from silkworms in 1901, as the cause of sudden-collapse disease of silkworms. The first use of Bt as a pesticide was in 1920. Now: Bt is the MOST-used biological control agent; over 200 registered Bt products marketed in the U.S. Bt is the biggest line of defense against the gypsy moth (*Lymantria dispar*, a forest pest that consumes foliage of 100’s of tree species)

We now know that there are thousands of strains of Bt, producing over 200 crystal (Cry) proteins that are active against an extensive range of insects and some other invertebrates. Farmers can be very specific in selecting the right Bt for their pest – less chance of non-target effects.

<table>
<thead>
<tr>
<th>Bt Strain</th>
<th>Effective against</th>
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<tbody>
<tr>
<td><em>Bt kurstaki</em> (Bik)</td>
<td>Lepidopterous insects (gypsy moth cabbage, looper)</td>
</tr>
<tr>
<td><em>Bt aizawai</em> (Bia)</td>
<td>wax moth larvae in honeycombs</td>
</tr>
<tr>
<td><em>Bt israeliensis</em> (Bti)</td>
<td>mosquitoes, blackflies, midges (Dipterans)</td>
</tr>
<tr>
<td><em>Bt san diego</em></td>
<td>certain beetle species; boll weevil (Coleopterans)</td>
</tr>
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</table>

In the 1980's use of Bt increased when insects became increasingly resistant to ALL of the synthetic insecticides (now, over 500 spp. are now resistant to one or more pesticides!). We were also realizing that pesticides were harming the environment and us.

<table>
<thead>
<tr>
<th>Synthetic Pesticide Class</th>
<th>Examples</th>
<th>Area of Effect on mammals (including humans)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organochlorines</td>
<td>DDT, toxaphene, dieldrin, aldrin</td>
<td>Reproductive, nervous, endocrine, and immune system</td>
</tr>
<tr>
<td>Organophosphates</td>
<td>Diazinon, glyphosate, malathion</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Carbamates</td>
<td>Carbofuran, aldicarb, carbaryl</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>Fenpropatrin, deltamethrin, cypermethrin</td>
<td>Poorly understood</td>
</tr>
</tbody>
</table>

How does Bt work?

During sporulation in some Bt strains, proteins are formed that aggregate to form a crystal. These protein crystals are toxic to very specific species of insects, but harmless to humans and the natural enemies of many crop pests (beneficial insects). There are more than 150 insects that are known to be susceptible in some way to Bt.

How did it all begin??

Numerous studies found that Bt products were:

**Safe for use in the environment**

**Nontoxic to mammals**

**Non-allergenic**

**Biodegradable**

**Target-specific (no known non-target effects)**

In fact, many countries in the world have incorporated Bt aerial spraying into their pest control program. Bt products are used on millions of acres of wooded areas and agricultural crops. These sprays are used to control for gypsy moths, blackflies, mosquitoes, and many other pests in forestry and urban areas.
With the advancement in molecular biology, it soon became feasible to move the gene that encodes the toxic crystals into a plant.

The first genetically engineered plant, corn, was registered with the EPA in 1995.

Today, GM (genetically modified) crops including, potato and cotton are planted throughout the world:

≥ 500,000 square miles of farmland planted to Bt crops.

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Classical Breeding vs. Genetic Engineering

**Traditional Plant Breeding**

DNA is a small chunk of genes, much like a small set of graphs.

*Traditionally, plant breeding overlaps many genes in one variety.*

**Plant Biotechnology**

Using plant biotechnology, we may add a single desired gene to a plant.

*Steady Gene + Commercial Plant Variety Improved Plant Variety*

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**How to make a GM crop**

1. Identify a useful trait (disease or pest resistance, enhanced food or fuel quality)
2. Identify gene(s) responsible for trait
3. Clone gene(s)
4. Modify gene(s): delete introns, alter codon makeup to accommodate host, hook to host-friendly promoter and terminator; promoter may be constitutive or tissue-specific or otherwise regulate gene expression

CaMV promoter from cauliflower mosaic virus already works in most plants

NOS terminator from nopaline synthase of Agrobacterium already works in most plants

CaMV and NOS are in 85% of GM plants

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**Examples of Promoter/Gene Combinations**

- **constitutive Bt**
  - T-DNA border
  - constitutive
  - Bt
  - T-DNA border

- **tissue-specific (plant) Bt**
  - T-DNA border
  - tissue-specific (plant)
  - Bt
  - T-DNA border
How do you transform plants?

Generate explants
Sterile tissue culture; callus generation

Insertion of DNA into cells via biolistics ("gene gun")

The first “gene gun” was derived from a real gun. Developed at Cornell; at the Geneva campus, "Father Gun" and "Son of a Gun" reside on the second floor of Hedrick Hall.

A little more sophisticated these days…

The "gun" consists of two small 6" x 7" x10" stainless steel chambers connected to a 2HP vacuum pump. When the technician flicks the switch on the outside of the second chamber, helium is released at 1000 psi. The blast ruptures a disk about the size of a nickel. The explosion of the disk releases a shock wave which travels 1 centimeter until it hits another disk, which is free to move. Attached to the front of that disk are microscopic tungsten particles 1 micron in diameter coated with thousands of DNA molecules. This disk travels another centimeter at the speed of a rifle bullet, roughly 1300 feet per second, and hits a screen, which detains the disk, but "launches" the microscopic particles toward the target cells. The particles penetrate the cells and release the DNA, which is diffused into the nucleus and incorporated by the chromosomes of the plant.

Agrobacterium-mediated transformation

What is Agrobacterium?

Agrobacterium causes crown gall in potato and other plants. It can be used as a vector for transfer of desirable traits from one plant to another.

How does Agrobacterium work?

Selection of transformants

Regardless of method, DNA must cross barriers:

- Cell Wall
- Cell Membrane
- Nuclear Membrane

No method is very efficient – must screen thousands of lines

- GFP
- β-glucuronidase (blue color upon adding substrate)
- Antibiotic resistance (kanamycin)

6 to 15 years from start to finish (marketable crop)
Is GM technology good or bad?

Like many things, the answer depends on the context.

The Regulatory Process for Transgenic Crops in the US

Transgenic crops are regulated at every stage of development, from research planning through field testing, food and environmental safety evaluations, and international marketing.

The major regulators of transgenic crops are:

- Institutional Biosafety Comm./Variety Release Comm.
- Animal and Plant Health Inspection Service (APHIS) of USDA
- Food and Drug Administration (FDA)
- Environmental Protection Agency (EPA)
- State regulations (ODA)
- WTO
- International agreements

...what sorts of things are they considering?

What species could possibly be contaminated by cross-pollination with wild relatives?

<table>
<thead>
<tr>
<th>Bt endotoxin (crystal protein) gene</th>
<th>Crop</th>
<th>Wild Relatives in U.S.</th>
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<tbody>
<tr>
<td>Cry3A gene</td>
<td>potato (Solanum tuberosum)</td>
<td>S. fendleri, S. jamesii, and S. pinnatisectum</td>
</tr>
<tr>
<td>BtCry</td>
<td>maize (Zea mays)</td>
<td>teosintes (Zea landraces) and Tritesum spp.</td>
</tr>
<tr>
<td>Cry1Ac</td>
<td>cotton (Gossypium spp.)</td>
<td>Gossypium spp., trees, shrubs, and weedy species in Hawaii and S. Florida</td>
</tr>
</tbody>
</table>

Thought to be most valid concern; others would not pollinate or be fertile due to biological or phenological constraints

Risks feared from Bt:

Cross-contamination of genes - Although unproven, genes from GM crops might potentially introduce the new genes to native species.

Proactive measure:

The EPA prohibits planting of Bt cotton in Florida and Hawaii, where wild relatives of cotton exist and could possibly participate in genetic exchange.

In other states, wild relatives of Bt-engineered crops are unlikely to cross-pollinate because of differences in timing of flowering, or would not yield sterile offspring because of differences in chromosome number.

Risks feared from Bt:

Resistance: The biggest potential risk to using Bt-crops is resistance. Farmers have taken many steps to help prevent resistance.

Invasiveness: Genetic modifications, through traditional breeding or by genetic engineering could potentially change the organism to become invasive.

Thanks to evolution, every pesticide, including a natural one, will eventually select for resistant insects.

Currently in the field, the diamondback moth is the only insect found to have developed resistance against Bt. The diamondback moth larvae feed on all plants in the mustard family, including canola, mustard, broccoli, and cabbage. The diamondback moth larva is resistance to proteins made by the Bt strain kurstaki.

The EPA requires farmers who use Bt as a pesticide spray to take precautionary measures to slow this process:

1. Alternate use of Bt with other pesticides
2. Crop rotations: if different crops are grown, they likely have different pests and Bt can even still be used as long as it’s a different strain or crystal protein.
Risks and Concerns

- Is eating food from transgenic crops a health hazard?
- Can antibiotic resistance genes used as markers in transgenic crops be transferred to pathogenic bacteria?
- Will pollen from transgenic crops contaminate non-transgenic crop varieties?
- Will herbicide-resistant GMO crops create “superweeds”?
- Do transgenic crops reduce biodiversity?
- Will insect pests become resistant to Bt toxins?
- Will the widespread adoption of transgenic crops lead to increased corporate control of the world’s food supply?

... what do you think???