Exploring central concepts of gene expression: What can worms tell us about human diseases?

Overview

Many human diseases result from genetic mutations. In some cases, it is possible to generate a "disease model" by inducing a similar mutation in the corresponding gene of a model organism, such as mice. Simpler organisms including *Drosophila* (fruit flies) and *C. elegans* (soil nematode worms) have also been used successfully as disease model organisms, and offer some benefits – such as the ability to rapidly do screens to look for genetic or pharmacological suppressors of disease mutations. Amazingly, ~65% of identified human disease genes have a counterpart in *C. elegans* (Sonnehammer and Durbin, 1997; Culletto and Sattelle, 2000). *C. elegans* is actively being used to study numerous pathologies including: obesity, Duchenne muscular dystrophy, Alzheimer's disease, Huntington's disease, and polycystic kidney disease.

Your main objective in this unit is to address the central question: Is *C. elegans* is a good model system for investigating the function of your assigned human disease gene? (see Fig. 1). Each lab group will work with a unique *C. elegans* gene that is homologous to a human disease gene. You will use a combination of **bioinformatics tools and primary literature** to gather more information about your gene's function in humans and worms, and to determine how the deletion affects the mRNA and protein produced from the gene.

You will also examine *C. elegans* using two common methods that scientists use to look at loss of gene function:

(1) **Analysis of worm strains with a genetic deletion** To start, your group will be given *C. elegans* worms that we ordered from labs maintaining a collection of mutant worms called a "worm library"; worms obtained from a worm library will have a genetic deletion in your assigned disease gene. You will carefully observe their phenotypes (physical traits and behaviors). You will do PCR (polymerase chain reaction) and gel electrophoresis to confirm that the deletion is present in the worm's genome.

(2) **Analysis of worms with gene function knocked down using RNA interference (RNAi)** Your group will do RNAi by feeding wild-type (WT) worms genetically engineered bacteria that produce double-stranded RNA designed to block or alter the expression of the disease gene of interest. You will carefully observe the resulting phenotypes of the progeny of these RNAi worms to determine if RNAi "phenocopies" (causes the same phenotype) as the deletion mutant, and speculate about why or why not this is the case. You will also do PCR and gel electrophoresis to compare the genotype of your RNAi worms with those of WT worms and your deletion mutant worms.

As the main assignment for this laboratory exercise, you will prepare an individual poster which provides your reasoning for why or why not you believe *C. elegans* is a good system for investigating the function of your assigned human disease gene. You will contrast your empirical observations with what you learned and predicted from the literature and bioinformatics tools, and use these data to support your conclusion. Using what you've learned about how loss of your gene's function alters the protein/cellular pathway responsible for your phenotype, you will speculate about why the deletion and RNAi strains yield the observed phenotypes. You will also propose experiments to further investigate the function(s) of your disease gene using *C. elegans* and/or other systems.

Learning Objectives: Concepts

Students will use their knowledge of the Central Dogma to understand:

- why *C. elegans* is a useful model system for biologists
- several ways to "knock-out" gene function resulting phenotypes, and why this is helpful for scientists
- how various tools are used to study gene function, including:
  - how PCR works to amplify a specific piece of DNA
  - how to use primers to direct DNA polymerase to specific sites on DNA for PCR
  - what plasmids are, and how they are used to create genetically engineered bacterial strains
  - the difference between genotype and phenotype
  - bioinformatics to gather, store, and analyze biological information

Learning Objectives: Techniques

Students will learn how to:

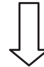
- become familiar with the physical characteristics of *C. elegans* and how to work with them in a laboratory setting
- use microtechnique – volumes that are very small
- extract DNA from *C. elegans*
- use PCR (repeated cycles of synthesis and melting) to amplify DNA
- prepare, run, and interpret electrophoretic gels to separate DNA fragments based on their size
- use aseptic technique when working with bacteria
- perform RNA interference on *C. elegans* using a feeding technique
- use several common bioinformatics tools to help compare nucleotide and protein sequences
- prepare a scientific poster summarizing your research

Terms you should understand

polymerase chain reaction • primer • nested PCR • cDNA • molecular weight standard • electrophoresis • ethidium bromide • allele • deletion allele • mutagen • promoter • intron • exon • 5' and 3' UTR (untranslated region) • gene splicing • mutagenesis • frameshift • phenotype • genotype • phenocopy • bioinformatics • RNA interference • feeding RNAi • plasmid • genetic engineering • transgenic • RNase III • T7 RNA polymerase • selection marker • TetR (tetracycline resistance) gene • AmpR (ampicillin resistance) gene • bacterial clone

"Is C. elegans a good model system for investigating the function of human disease genes?"

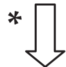
Identify worm genes that are homologous to disease genes in humans
(use bioinformatics tools and primary literature)



How we study loss of gene function specific for human disease

Obtain worms with genes mutated via deletions
(order deletion strains from labs maintaining worm libraries)

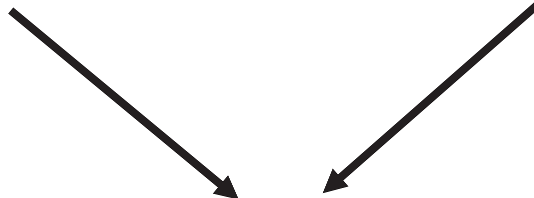
Generate worms with genes knocked down via RNAi
(feed WT worms genetically engineered bacteria)

* 

Observe phenotypes
(Confirm genotype using PCR & gel electrophoresis)

*

Observe phenotypes
(Confirm genotype using PCR & gel electrophoresis)



Compare phenotypes and genotypes of 3 strains (WT, gene deletion, and RNAi worms)
(contrast your observations with what you learned/predicted from the literature & bioinformatics tools)

Use information you obtain to address the central question and summarize in your poster

***Assumption:** altered protein/cellular pathway resulting in altered gene expression responsible for phenotype

Figure 1 : Organizational chart for 4-week molecular genetics unit

Background

C. elegans as a model system for studying basic biological processes and disease

C. elegans is a non-parasitic soil nematode that is widely used as a model system for studying numerous cellular, physiological, and disease processes. There are over 200 laboratories world-wide that use *C. elegans* in biomedical research. *C. elegans* has several features that make it particularly useful as a model system, including the following:
1. Ease of culture. *C. elegans* thrive at room temperature and can be grown in Petri dishes seeded with *E. coli* bacteria, which is one of the worm's food sources. They can survive prolonged periods of starvation and can be frozen for storage. Adults are only ~1 mm long, so large numbers (~10,000 / 6 cm Petri dish) can easily be generated and stored.
2. Fast life cycle. *C. elegans* only take approximately 3 days at 20°C to go from being a fertilized egg to a fertile adult. There are four larval stages before they become adults. Under optimal conditions, *C. elegans* will live for 2-3 weeks. The *C. elegans* life cycle and images of different stages are shown in Appendix B.
3. Reproduce as hermaphrodites. *C. elegans* exist predominantly as hemaphrodites, and rarely as males. Hermaphodites self-fertilize themselves. This facilitates genetics because a single worm can create numerous clones of itself by self-fertilizing.
4. Large brood size. One *C. elegans* hermaphrodite produces approximately 300-350 progeny, and even more if it is fertilized by a male.
5. Transparency and simple body plan. *C. elegans* embryos and worms are transparent and have a simple body plan (refer to http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm), facilitating visualization of cells and tissues. *C. elegans* has nerves, muscles, epithelial, and endothelial cells that develop and function in similar ways to their human counterparts.
6. Defined Lineage. *C. elegans* have about 1000 somatic (non-germline) cells and the lineage for each is known (i.e. when and where it was born and what cells it was derived from). The development of wild-type *C. elegans* embryos is strongly determinative – it proceeds the same way each time.
7. Strong genetics. *C. elegans* was the first multicellular organism to have its entire genome sequenced (in 1998). Its genome has about a hundred million base pairs, which is 20x bigger than *E. coli*, but only 1/30 the size of the human genome. Deletions in its genes can be generated by mutagenesis (using chemical agents or radiation to alter DNA) and then identified by gene mapping and sequencing. Gene function can be reduced using RNA interference (described below). Engineered DNA can be introduced into the germline to generate "transgenic" worms.

Although *C. elegans* are a lot simpler than humans, their tissues develop, function, and can become diseased in similar ways. Furthermore some major signaling pathways (cell communication pathways) that control processes like cell division, differentiation, and programmed cell death (apoptosis) are remarkably well conserved between *C. elegans* and vertebrates. As mentioned above, *C. elegans* is actively being used as a model system to investigate numerous different diseases.

Bioinformatics

The amount of data generated in science is continually increasing. Endeavors like the human genome sequencing project have left us with enormous, almost unmanageable amounts of information. Bioinformatics is a field that combines computer science and biology – its goal is to develop technology to facilitate the management and analysis of this huge amount of data.

As we did in the enzyme unit to study alkaline phosphatase, we will use bioinformatics to gather information and for analysis. There are numerous web-based bioinformatics resources that you will be using to find out more about your group's gene. You are familiar with some in the list below, but others will be new:

1) Wormbase. **http://www.wormbase.org** This database of the model organism *C. elegans* and related nematodes has a page for each of the 19,000 genes in *C. elegans*, with links to the genome sequence, similar genes in *C. elegans* in other organisms, all the publications mentioning the gene, as well as links to other databases.
2) Simple Modular Architecture Research Tool: SMART. **http://smart.embl-heidelberg.de** SMART allows the identification and annotation of protein domains.
3) National Center for Biotechnology Information: NCBI. **http://www.ncbi.nlm.nih.gov** The NCBI describes itself as a: "national resource for molecular biology information, NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and

disseminates biomedical information - all for the better understanding of molecular processes affecting human health and disease." NCBI has multiple interlinked databases.

4) Basic Local Alignment search tool: BLAST. **http://www.ncbi.nlm.nih.gov/Sitemap/ResourceGuide.html#BLAST** This algorithm is used to find regions of homology or similarity in nucleotide and protein sequences.
5) Online Mendelian Inheritance in Man: OMIM. **http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM** A catalog of human genetic diseases and associated genes.
6) PubMed. **http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed** This is a database of abstracts from the biomedical literature

RNA interference

The 2006 Nobel Prize in Physiology or Medicine was awarded to Andrew Fire and Craig Mello for their discovery of RNA interference. RNA interference, called RNAi, is both a powerful laboratory technique for knocking down gene function and a natural process used for viral defense and gene regulation.

Please view these web resources on RNAi:

Nova, Science Now – RNAi tutorial
http://www.pbs.org/wgbh/nova/sciencenow/3210/02.html

"Watch the segment" is about 15 min. long, but it is an excellent intro that is well worth your time!
"RNAi explained" is also excellent, please watch after the intro.

Howard Hughes Medical Institute – RNA interference tutorial
http://www.hhmi.org/biointeractive/rna/rnai/index.html

This is also very good and should be viewed after the two Nova segments.

The discovery of RNAi

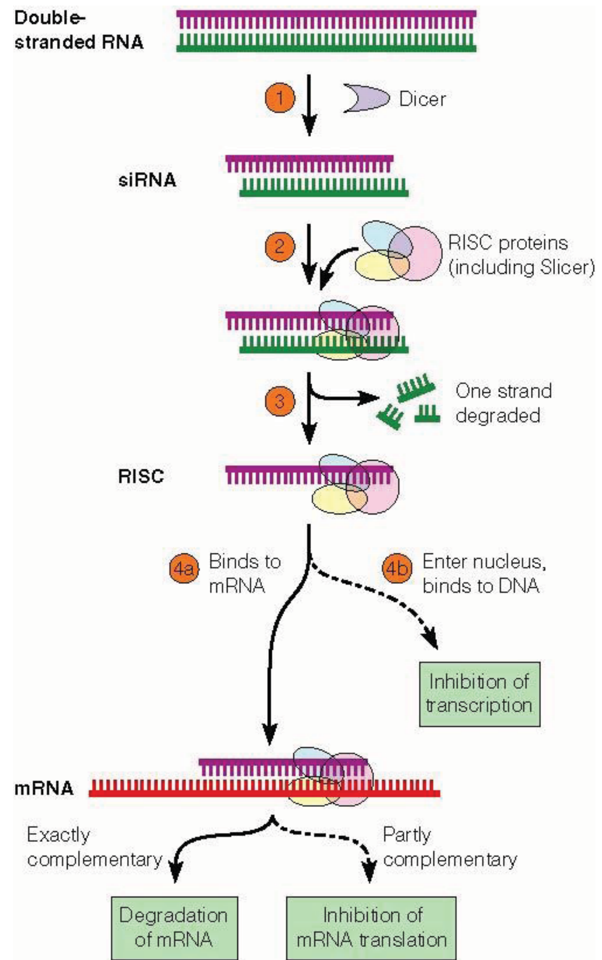
Fire, Mello and colleagues were working with the *unc-22* gene in *C. elegans* when they stumbled upon RNAi (Fire et al., 1998; reviewed in Mello and Conte, 2004). A deletion in *unc-22* makes worms twitch excessively, because the gene product of *unc-22* is an important muscle protein. The researchers were using treatment with large amounts of single stranded antisense RNA to attempt to knock down expression of *unc-22* in wild-type *C. elegans*. At the time, antisense RNA was believed to work through binding to the corresponding mRNA and preventing it from being transcribed. They found that worms injected with *unc-22* antisense RNA exhibited only weak twitching. They and others had tried to use single stranded sense RNA as a negative control, but surprisingly, they found that the sense strand gave the same mild twitching that they saw with the antisense RNA. Since both antisense and sense RNA could knock down gene function, they concluded that the effects of antisense RNA were not due simply to binding to and soaking up mRNA. They knew that the sense and antisense RNA had one thing in common: that they were generated in the lab. Was there something special about the lab-generated RNA they were introducing to their worms? Could the worm cells recognize this and respond?

Fire, Mello and colleagues knew that RNA made in the lab is not completely pure and can have small levels of contaminating complementary strands. So they increased the amount of complementary strands, made purified double stranded RNA (dsRNA), and injected it into worms. They saw a very potent knock-down effect: the worms twitched excessively – they had discovered RNAi. Fire and Mello also calculated that only a few molecules of dsRNA per cell were enough to see an effect. This was much less than the amount that would be needed to soak up all of the thousands of *unc-22* mRNAs in each muscle cell. Thus, small levels of dsRNA have an amplified effect in the cells. Further, their injections were into the gonad of the worm and the dsRNA effect spread to muscle and other cells. Thus, the effect of dsRNA is both amplified within cells and can spread to other tissues. As explained in the genetic deletion section (page 4), blocking the function of a gene can be very useful in understanding its function. RNAi gives us one more tool to understand gene function.

How RNAi works at the molecular level

How RNAi works, exactly, is still being worked out. Each organism has several variations of the mechanism. We'll use the model in your textbook:

Figure 2: RNA interference by double stranded RNA. This is Figure 23-35 from *World of the Cell* (Becker et al., 2006). When a cell encounters double stranded RNA, (1) the enzyme Dicer cleaves the double-stranded RNA into siRNAs (small interfering RNAs) about 21-22 base pairs in length. The resulting siRNAs (2) combine with RISC proteins and (3) one of the two RNA strands of the siRNA is degraded. The remaining strand then binds via complementary base pairing to a target mRNA molecule in the cytoplasm (4a) or to a target DNA sequence in the nucleus (4b), thereby silencing gene expression at either the translational or transcriptional level. The most common situation (indicated by the solid arrows) is an exact complementary match between the siRNA and a corresponding mRNA, which triggers mRNA degradation by Slicer, an enzyme component of the RISC.



Biomedical uses for RNAi

RNAi is now used in labs around the world as a technique to knock-down gene function. Numerous organisms are susceptible to RNAi including plants, worms, fruit flies, mice, and humans. There are even hopes that RNAi may be an effective way to treat some human diseases. Different organisms differ in the type of dsRNA template and the mode of delivery necessary to elicit an RNAi effect. In *C. elegans* dsRNA (typically 500 bp – 3 kB in length) is effective at inducing RNAi. The dsRNA can be delivered by three different methods: 1) injection of purified dsRNA, as was done by Fire and Mello; 2) soaking worms in a liquid solution containing dsRNA; and 3) by feeding worms bacteria that are induced to make dsRNA. We will use feeding RNAi in this laboratory exercise.

Feeding RNAi in *C. elegans*

Amazingly, RNAi can be accomplished in *C. elegans* by feeding them *E. coli* engineered to produce the desired double stranded RNA (Timmons and Fire, 1998). The bacterial strain used for feeding RNAi, HT115(DE3), is genetically engineered to work for you. This is a non-pathogenic lab strain with some special features that are described below. In this laboratory exercise, you will induce these bacteria to produce a specific sequence of double stranded RNA (dsRNA). When the worms eat this *E. coli,* it will generate an RNAi response that results in degradation of corresponding mRNAs. In this way you can knock-down the activity of your worm gene of interest. Sometimes RNAi will result in all (or most) of the mRNAs being degraded and this results in a null phenotype. Other times only some of the mRNA is degraded, which can result in a weaker phenotype.

If you could look at the genome of *E. coli* strain HT115(DE3) with a super-powerful microscope or if you sequenced its genome, and compared it to wild-type, you would see that the gene for ribonuclease RNase III has

been removed and replaced by two alternate genes. These are genes encoding T7 RNA polymerase and TetR. A schematic of the generation of the genetically-engineered *E. coli* strain HT115(DE3) is shown in Figure 3.

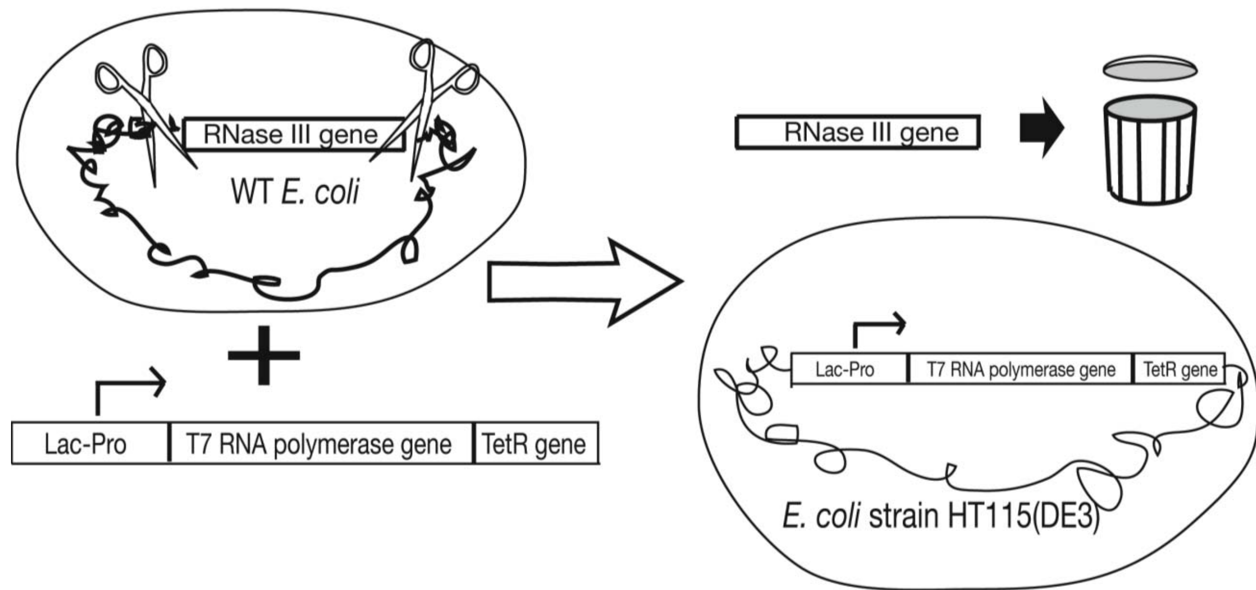


Figure 3: Schematic showing genetically engineered E. coli strain HT115(DE3). This is the bacteria used for feeding RNAi. "WT" = wild-type. Scissors represent restriction enzymes that are used to cut the genomic DNA of the bacteria. The rectangles represent DNA regions we will be discussing, and the curly lines represent other DNA. The left side of the diagram shows the removal of the RNase III. Removing RNase III will render the *E. coli* incapable of degrading dsRNA (a process normally used by the *E. coli* to prevent attack from invading foreign dsRNA). Since this strain of *E. coli* cannot degrade dsRNA, the dsRNA that is generated within the bacteria will not be destroyed. Thus, we can use this strain to make dsRNA for feeding RNAi.

Also shown on the left side of Figure 3 is a rectangle representing the T7 RNA polymerase gene driven by a lactose-responsive promoter (Lac-Pro), and the TetR gene. In HT115(DE3), this group of genes has been inserted into the *E. coli* genome at the site where the RNaseIII gene was removed. When these *E. coli* encounter lactose, the T7 polymerase gene will be expressed. How do researchers know whether this *E. coli* strain has incorporated the gene of interest? They use a selection marker, in this case a gene that conveys tetracycline resistance, called TetR. The TetR gene is constitutively (always) expressed and allows for the engineered *E. coli* strain to resist being killed by the antibiotic, tetracycline. This allows researchers, like you, to make sure the bacteria you grow in lab does not lose the foreign DNA. As long as your bacteria survive in the presence of tetracycline, you can be fairly certain that the bacteria contain your foreign gene.

These bacteria need one more thing to be able to induce RNAi when eaten by worms: instructions for making the dsRNA of interest. These instructions are in the form of an engineered piece of DNA, called a plasmid, that is added to the *E. coli* strain HT115(DE3). Plasmids are circular piece of DNA that are replicated with each round of bacterial division. The plasmid contains DNA matching your gene of interest flanked by two T7 promoters. Thus, the strains you will receive contain two pieces of foreign DNA. One is stuck into the genome and another one is in a plasmid (refer to Figure 4).

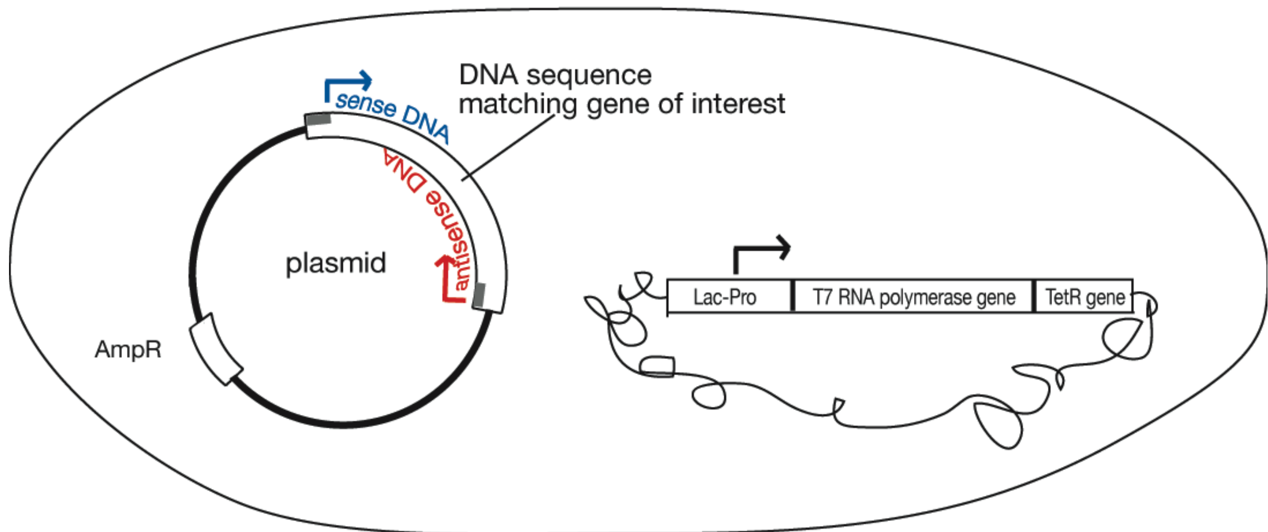


Figure 4: Diagram showing *E. coli* strain HT115(DE3) with a plasmid for feeding RNAi. This bacteria has foreign DNA integrated into the genome and also has a plasmid. All HT115(DE3) bacteria have DNA integrated into their genome that permits expression of T7 RNA polymerase when they are exposed to lactose (refer to Figure 3). HT115(DE3) bacteria can have different plasmids, which allows the bacteria to produce dsRNA against different targets (refer to Figure 5 for more specifics). Note that this diagram is not to scale. In particular, the genomic DNA is much, much longer than the plasmid DNA. The large oval represents one *E. coli* feeding bacterium. In reality you will work with billions of identical *E. coli* (a clone population) that will be fed upon by *C. elegans* for each condition in your experiment.

The following describes in more detail the role of the plasmid DNA in enabling the **bacterial clone** to be used for feeding RNAi.

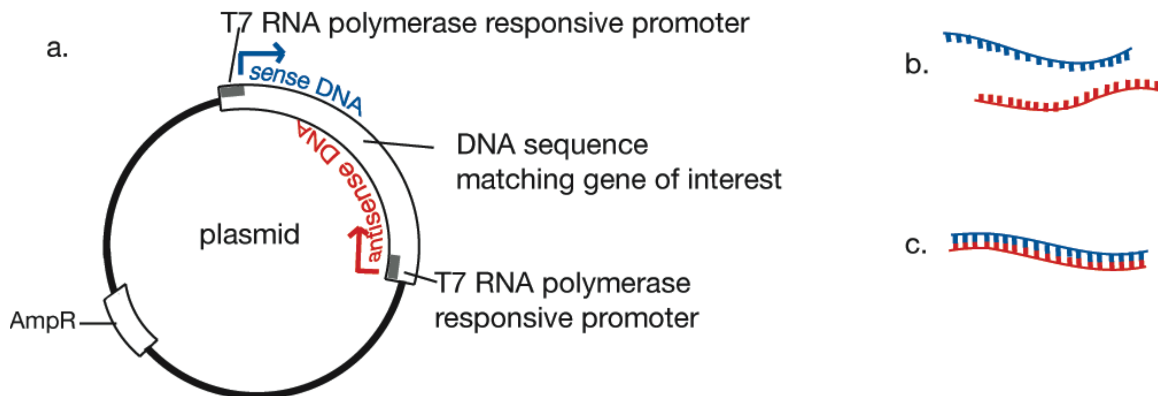


Figure 5: Schematic of feeding RNAi plasmid. This plasmid is also shown in Figure 4. T7 RNA polymerase will result in the production of many copies of double-stranded RNA (dsRNA).

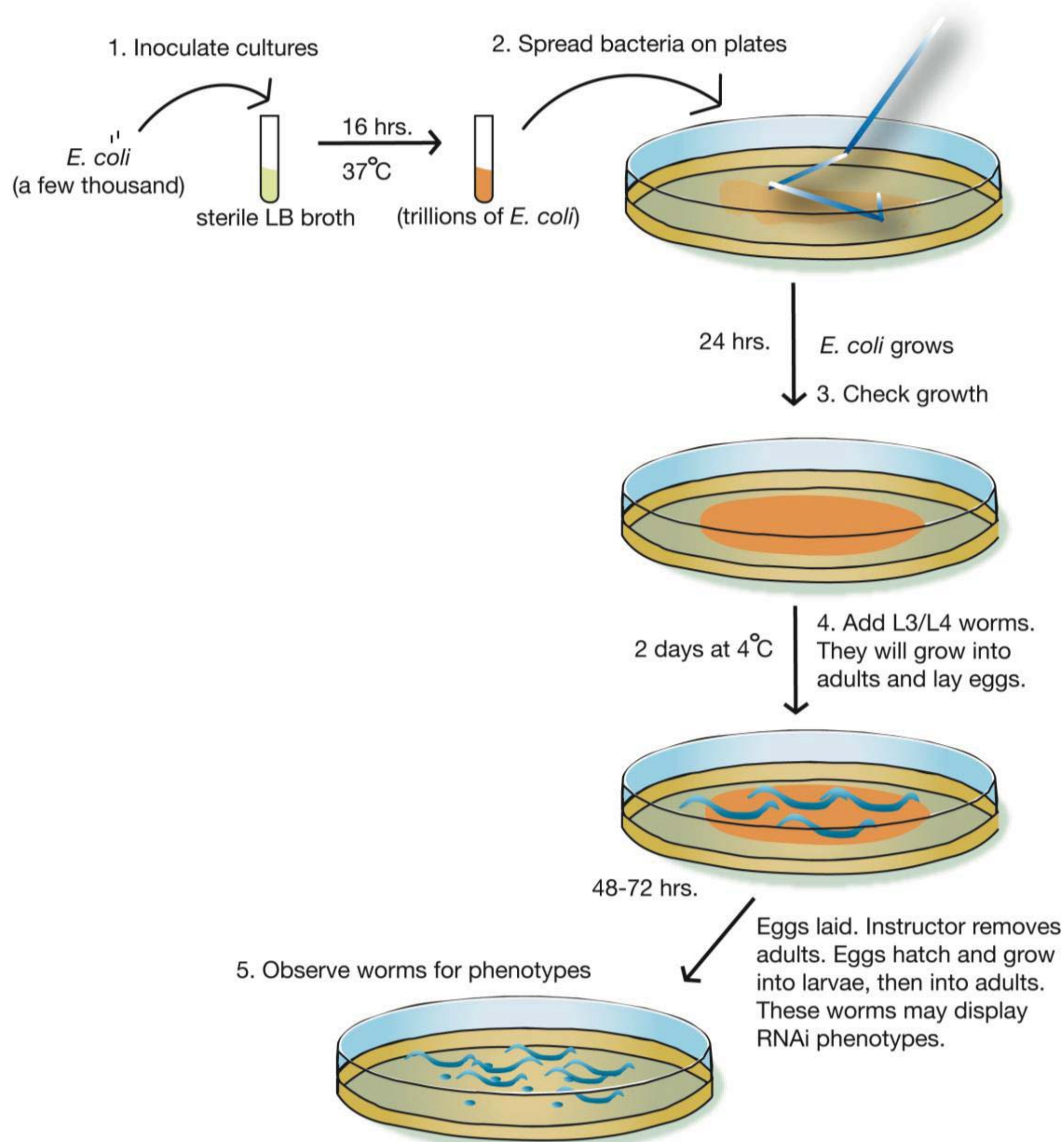
In the presence of lactose, T7 RNA polymerase will be produced by the foreign DNA in the bacteria's genome. This T7 polymerase will bind to the T7 promoters on the plasmid and prime production of RNA (Figure 5a). Thus, you'll end up with single stranded RNA corresponding to both the sense and antisense strands (Figure 5b) that will then anneal to form dsRNA (Figure 5c). This is in contrast to normal gene transcription where the promoter is only found upstream of the gene and only one piece of ssRNA (mRNA) is produced.

Similar to the tetracycline resistance gene in the previous diagram, the AmpR, or ampicillin resistance gene is present in this plasmid as a selection marker. As long as your bacteria survive in the presence of ampicillin, you can be fairly certain that the bacteria contain the plasmid.

When the worms eat HT115(DE3) *E. coli* bacteria carrying a feeding RNAi plasmid, the dsRNA produced by the bacteria will be absorbed by the worm's intestine. Although it is not understood exactly how this happens, RNAi is systemic in *C. elegans*, meaning that it will spread to all the worm's cells (including the eggs it is making). This leads to destruction of mRNAs throughout the worm (and in its eggs) that match the sequence of DNA in the feeding RNAi plasmid. An overview of the feeding RNAi protocol is shown in Figure 6.

Pause and Ponder: What do you think will happen to your gene of interest when it is targeted by RNAi? Will any protein be made from it?

Figure 6: Overview of Feeding RNAi Protocol.



1. Inoculate cultures (Week 7): Instructor inoculates genetically engineered *E. coli* cultures that are allowed to grow overnight with shaking at 37°C.

2. Spread bacteria on plates (Lab Week 7): Students spread bacteria on feeding plates containing lactose, ampicillin, and tetracycline (Appendix A). Grow for 24 hours at room temperature.

3. Check growth: Instructor checks for bacterial growth and places plates at 4°C for storage.

4. Add worms (three days before Lab Week 8): Instructor warms up the plates to room temperature and adds larval worms to feed on the bacteria.

5. Observe worms (Discussion & Lab Week 8): Students observe worm progeny and note phenotypes.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction involves using cycles of DNA replication to amplify a specific part of a DNA molecule. A typical PCR reaction can generate over a billion copies of DNA from just one copy of the starting DNA template. The ability to amplify a particular piece of DNA depends on having two primers (short single-stranded pieces of DNA), that flank the region to be amplified. One primer is designed to anneal to the 3' end of one strand, and one primer is designed to anneal to the 3' end of the opposite, complementary strand. Primers provide a free 3' OH group which allows DNA polymerase to add more nucleotides. DNA polymerase mediates replication in the 5' → 3' direction.

A cycle consists of:

1. "Melting" double stranded DNA containing the target sequence into single strands by heating it to near 100°C.
2. Annealing the primers to their complementary sequences (50-65°C).
3. Extending DNA from primers using DNA polymerase and four nucleotide triphosphates (72°C).

This cycle is repeated many times, each time doubling the amount of target DNA. The whole process can be automated, and necessitates using a heat-stable DNA polymerase (Taq polymerase) isolated from a bacterium (*Thermus aquaticus*) that normally grows in hot springs.

The following animations will help you to understand the processes of PCR and aid in answering the pre-lab questions:

The Polymerase Chain Reaction by Sumanas, Inc.
http://www.sumanasinc.com/webcontent/anisamples/molecularbiology/pcr.html
Polymerase Chain Reaction by the Dolan DNA Learning Center
http://www.dnalc.org/ddnalc/resources/pcr.html

When working with genomic DNA, like we are in this exercise, it is often necessary to perform **nested PCR**. This is a technique that is used when working with a large complex mixture of DNA. Nested PCR involves using two rounds of PCR. Using two round of PCR helps to provide a higher level of amplification of the desired target, and also helps to increase specificity. For the first round (external PCR), a slightly larger piece than the region of interest is amplified. DNA generated in the external PCR reaction is then used as template for performing PCR to amplify the region of interest (internal PCR). This is shown schematically in Figure 1 on the next page. One benefit of this approach is that the two rounds of PCR yields more amplification than a single round – which helps in the detection of targets that might be present in relatively few copies in the starting DNA. Another benefit of this approach is that, if the wrong locus were amplified by mistake in the first round of PCR (due to primers binding to sequences besides the target – which is a possibility when working with a complex mixture of DNA), the probability is very low that it would also be amplified a second time by the second pair of primers.

Analysis of DNA by Electrophoresis on Agarose Gels

Another important technique in DNA technology is the ability to separate DNA fragments by means of agarose gel electrophoresis. This allows scientists to analyze the size of different DNA fragments in a solution. DNA is loaded into sample wells at the top of an agarose gel and then a voltage is applied across the gel. The negatively charged DNA migrates towards the positive pole. The agarose acts like a sieve, and the size and configuration (linear, circular, or supercoiled) of the DNA affects its migration through the gel. In this lab we will be working with DNA generated by PCR, which is linear. Small DNA fragments travel faster than large ones because they pass more easily through the pores in the gel. The molecular weight of linear DNA fragments can be determined by comparing the distance they migrate with a series of linear markers of known molecular weights run in the same gel. Series of linear markers of known molecular weights are available commercially and are often referred to as molecular weight markers, or a DNA ladder.

The DNA bands are made visible by staining the gel with ethidium bromide, a dye that intercalates between the stacked bases of DNA. The ethidium bromide-DNA complexes fluoresce to give a pinkish-orange color when irradiated with UV light. On photographs, the bands appear white on a black background. (Note that ethidium bromide is a powerful mutagen and must be used with care. You MUST WEAR GLOVES when handling gels containing ETHIDIUM BROMIDE!) As mentioned above, comparing experimental unknowns with a DNA ladder allows determination of the molecular weights of unknown fragments.

This is a nice animation that will help you understand gel electrophoresis:

Gel Electrophoresis by the Dolan DNA Learning Center
http://www.dnalc.org/ddnalc/resources/electrophoresis.html

Detection of Genetic Deletions with Nested PCR and Agarose Gel Electrophoresis

A powerful technique for analyzing gene function is the production and analysis of genetic deletions. Analyzing the phenotype induced by a deletion in a gene can help us understand that gene's function. Furthermore, genetic deletions help scientists to understand the molecular mechanisms regulated by the affected gene. Depending on where the deletion occurs, it can have different effects. Mutations, such as deletions, can result in loss-of-function of the affected gene. This can involve a complete loss-of-function (exhibited by null alleles) or only a partial loss-of-function. [In genetic systems, a **null allele** is an allele that cannot produce a normal gene product at the molecular level, or an allele that results in total absence of normal function at the phenotypic level.] *C. elegans* homozygous for a null allele of *unc-52** die during embryogenesis with failed muscle development, whereas *C. elegans* homozygous for a weak loss-of-function allele for *unc-52* develop into adults that eventually become paralyzed. Whether a deletion causes a null phenotype or a weaker loss-of-function depends on the gene structure and where the deletion occurs. For instance, if the start codon(s) for a gene are deleted, this will generate a null mutation since the gene will not be able to produce an mRNA transcript, and hence will not produce protein. Deletions may affect genes in other ways. For instance a deletion could result in a frameshift, affect splicing, or eliminate regions that encode important functional domains. These situations would likely result in the production of an aberrant protein. * In *C. elegans*, the names of genes are italicized (*unc-52*) and the names of proteins are in capitalized (UNC-52).

The deletion strains that we will be working with were generated by the *C. elegans* Gene Knockout Consortium, a group that is striving to generate genetic deletions for every *C. elegans* gene. Various treatments can be used to induce deletions, and the ones we are working with were all generated by treating worms with trimethylpsoralen (TMP) and UV irradiation. To detect and track genetic deletions, scientists often use nested PCR, coupled with agarose gel electrophoresis. Primers are designed that flank the region of interest. For wild-type transcripts, a product of certain length will be produced. If a deletion is present in this region (but the binding sites for the primers are still intact) then the product produced from the PCR will be smaller than the wild-type product. This is shown schematically in Figure 7.

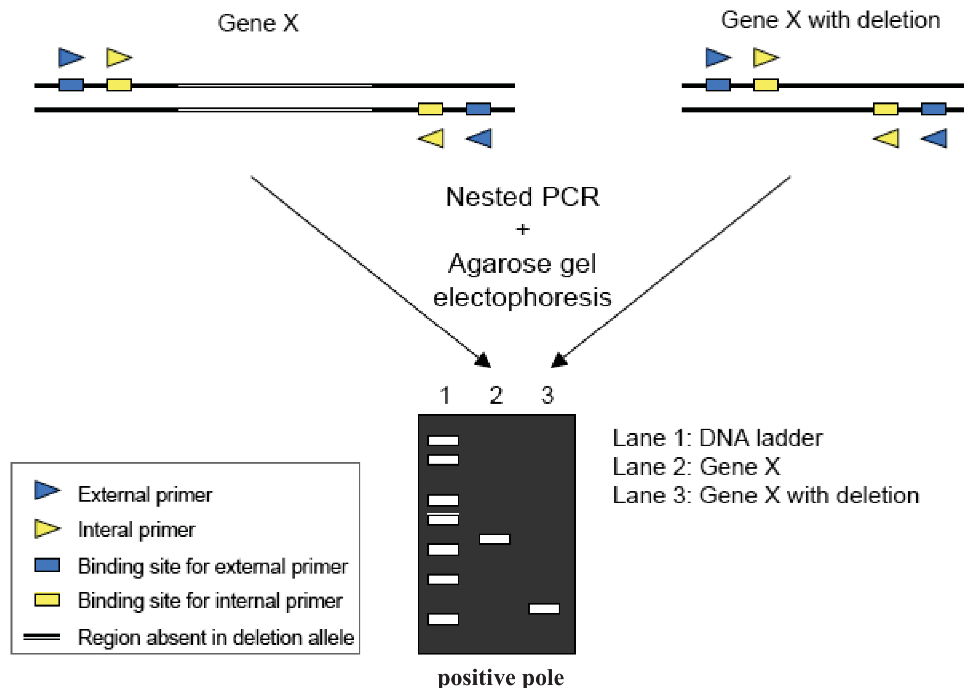


Figure 7: Nested PCR. As described in the text above, nested PCR involves using two rounds of PCR to amplify a region of interest, and is often used when the starting DNA pool is large and complex. Shown are gene x and gene x

with a deletion in the indicated region. External and internal primer sets are shown that flank the deleted region. When the products from the nested PCR are run on a gel, the presence of the deletion can be detected.

Pause and Ponder: Why does the DNA is lane 3 run faster than the DNA in lane 2? Can this technique be used to determine the size of a deletion? (If so, how?)

****

Schedule

This lab will take 4 weeks (7 sessions) to complete:

Week 7: **Discussion**
--General overview, discussion of *C. elegans*
--Observe WT and deletion strain *C. elegans* (Procedure A).
--Instructor inoculates bacterial cultures (Procedure B).

In this week you will get an introduction to this lab unit. Your group will be given wild-type C. elegans *and* C. elegans *with a deletion in a human disease gene. You will observe these worms and* ***will need to take good notes (and draw pictures or take images / movies) to document their phenotypes to include on your poster****.*

Week 7: **Lab**
--General overview, discussion of RNAi and begin RNAi experiment.
--Observe WT and deletion strain *C. elegans* (Procedure A).
--Plate bacteria from cultures on feeding plates (Procedure B).

There will be a discussion of RNAi and your instructor will have inoculated the bacterial cultures needed for your experiment. This required using aseptic technique.

Week 8: **Discussion**
--RNAi Pre-Lab is due.
--Observe and characterize phenotypes induced by RNAi and deletion mutation.
--Read “Supermodels” paper before lab

Week 8: **Lab**
--PCR / Deletion Screening Pre-Lab is due
--Discuss model systems
--Observe and characterize phenotypes induced by RNAi and deletion mutation.
--Isolate DNA from *C. elegans* (Procedure D).
--Discussion of PCR.
--Design primers to detect the deletion in your gene of interest (Procedure C).

You will observe and document the phenotypes induced by RNAi in your experiments. ***You will need to take good notes (and draw pictures or take images / movies) to include on your poster.***
Your group will isolate DNA from the 3 strains of C. elegans *(WT, deletion strain, and RNAi worms) to serve as a template for nested PCR next week. Your group will also complete a worksheet (check assignment) to choose primers for doing nested PCR to detect your deletion. .*

(Spring Break)

Week 9: **Discussion**
--Check PCR primers
--Perform external PCR (Procedure E).
--Discuss model systems & poster expectations

Your instructor will let you know if your group has selected the right primers. There will also be a discussion on bioinformatics. Your group will begin doing nested PCR to detect the deletion in your gene of interest. This will involve setting up external PCR reactions using the DNA isolated in lab just before spring break.

Week 9: **Lab**
--Perform internal PCR (Procedure E).
--Discuss bioinformatics.
--Start bioinformatics analysis on your gene.

Your group will continue the nested PCR experiment to detect your deletion by setting up internal PCR reactions in lab using your external PCR reactions from discussion as a template. Using the bioinformatics worksheet as a guide, you will begin bioinformatics analysis on your gene of interest. ***Some of the data from your bioinformatics analysis should be included on your poster, and this is described in more detail on the Mini-Poster Guidelines Handout****.*

Week 10: **Discussion**
--Continue work on Bioinformatics investigation.
--Work on posters (draft due in lab).

Week 10: **Lab**
--Analyze PCR products by agarose gel electrophoresis (Procedure F).
--Peer review of mini-poster draft
--Gel electrophoresis trouble shooting worksheet

Your group will analyze your PCR products from the nested PCR performed last week, using agarose gel electrophoresis. There will be a worksheet about agarose gel electrophoresis (check assignment) to do with your group while the gels are running. ***You will need a picture of the gel for your poster****. In free blocks of time, please continue to work on your Bioinformatics investigation of your gene.*

Draft of RNAi mini-poster is due in lab week 10.
Completed poster + GEA form due in lab week 11.

Procedures

A. Observe worms (Week 7).

Use your microscopy skills to observe wild-type worms and worms that have the deletion in your assigned gene. Take photos (or movies) and / or draw and write down what you see. This is important information to include on your poster.

How do the worms with the deletion compare to wild-type worms? Do they look similar? Do they move and behave the same? Do they have the same size, shape, and appearance? If there are any differences, approximately what percentage of the worms is affected? Here are some common phenotypes to look for:

unc = uncoordinated
slu = sluggish
prl = paralyzed
rol = roller (roll instead of moving normally)
emb = embryonic lethal
lvl = larval lethal
adl = adult lethal
dpy = dumpy (short and stubby)
bmd = body morphogenesis defects (misshapen)

lon = long
sma = small
clr = clear
gro = growth defective
pvul = protruding vulva
him = excess males (males usually occur only rarely on plates)

Movies of some of these phenotypes are available at: http://www.wormclassroom.org/ge.html
Pictures of wild-type worms are shown in Appendix B.
Some worm plates will be available for viewing that exhibit some of the phenotypes listed above.

B. RNAi experiment (Week 7, 8).

RNAi is a powerful technique for knocking-down gene function (refer to pages 5-6). Your group will use feeding RNAi (refer to pages 7-10) to knock-down your disease gene of interest in wild-type worms. You will determine if feeding RNAi yields a similar or different phenotype than that observed with worms carrying a genetic deletion in that gene (observed previously in Week 7). Information from your bioinformatics analysis should allow you to speculate why you see the phenotypes that you do with the deletion and with RNAi. This is important information to include on your poster.

1. Inoculate cultures (done by instructors before Week 7 lab).

We will work with plates that have colonies of the feeding bacteria you will need for your experiment. We have the following bacteria: (1) HT115(DE3) containing an empty feeding plasmid (negative control); (2) *E. coli* with no feeding plasmid (negative control, should not be able to grow feeding plates, which contain ampicillin); (3) HT115(DE3) carrying a feeding RNAi plasmid for your gene of interest; and (4) HT115(DE3) carrying a feeding RNAi plasmid for *sma-1* (positive control, should yield "small" worms).

Always wear gloves and use aseptic technique when working with bacteria. *Protocol used by instructors:*

a. Label culture tubes with date and type of bacteria.
   (Tubes contain 3 mL of LB (Appendix A) with antibiotics.)
b. Using a Bunsen burner, sterilize the end of a metal loop and wait several seconds until cool.
c. Carefully touch cooled loop to one colony on plate.
d. Take lid off culture tube, flame top of tube, and swish loop around in culture media.
e. Flame top of tube again and put lid back on.

Note: be careful not to contaminate the lid of the culture tube. You can either hold it while you inoculate the culture, or place it bottom side up in a clean, safe spot.

2. Plate bacteria on feeding RNAi plates (Week 7 lab).

a. Mix bacteria cultures well. Add 75 μL of each culture to the middle of a 6 cm dish.
b. Sterilize an L-shaped glass spreader using a Bunsen burner (lab instructor will demonstrate).
c. Cool the spreader on an unseeded plate.
d. Spread the bacteria into a lawn (to about a half inch from the sides of the plate).
e. Plates will be allowed to dry, and bacteria will grow into a lawn within 24 hours.

The instructor will add larval worms to the feeding RNAi plates 2-3 days before week 8 lab. The larval worms will grow into adults and produce offspring with the RNAi effect. For worms fed the empty feeding plasmid, we expect that their will be no RNAi effect: the eggs will hatch, and the larval worms will mature into normal adults. These normal developmental processes may be perturbed in the experimental conditions.

3. Observe phenotypes induced by RNAi (Week 8 discussion & lab).

Observe the progeny of worms placed on the plates last week by an instructor. Compare the phenotype obtained with RNAi to that exhibited by WT worms and worms with the deletion allele. Is the phenotype the same, weaker, or more severe? Are different phenotypes present in the RNAi worms that weren't exhibited by worms with the deletion? Use the notes and images / drawings that you generated from your observations in week 7 as a guide.
These observations are important to include on your poster.

C. Choose primers to detect a deletion in your gene of interest (Week 8).

Use the information presented on the PCR Primer Selection Worksheet (handout), to select an external and internal primer set that will allow you to detect the deletion in your assigned disease gene of interest.

D. Extract DNA from *C. elegans* (Week 8).

Use the following procedure to collect DNA from wild-type worms, RNAi worms, and worms carrying the deletion in your disease gene of interest. This DNA will be used as a template for PCR next week.

a. Add 500 µl of sterile H_2O to a full or starved 3.5 cm plate of *C. elegans*.
   (Do this gently, try to minimize disturbing any bacteria on the plate)
b. Swirl plate and then collect 50 µL and put in a PCR tube.
c. Add 50 µL PK buffer (Appendix A).
d. Put in a –80°C freezer for 15 min or longer.
e. Put tube in thermocycler and run Worm Lyse program:
   65°C 4 hours
   95°C 15 min.
   4°C forever

E. Perform nested PCR to detect deletion of interest (Week 9).

Performing nested PCR on the DNA from wild-type worms and worms carrying the deletion in your disease gene of interest will allow you to “see” the presence of the deletion when you run out the DNA on an agarose gel next week. You will also include DNA from your RNAi worms (will this be more similar to WT or deletion strain worms?). As mentioned above, performing nested PCR involves doing two rounds of PCR (an external reaction and an internal reaction). Refer to page 11 and 12 for a detailed explanation of nested PCR.

1. Set up <u>external PCR reactions</u> on samples.

Use the following as templates for your reactions: (1) DNA collected from wild-type worms (positive control), (2) DNA collected from worms with the deletion in your disease gene of interest, (3) DNA collected from your RNAi worms, and (4) sample with no DNA (negative control).

<u>Important:</u> to avoid contaminating your samples with unwanted DNA, wear gloves at all times.

a. Dilute lysed worm solution (collected last week) 1:10 (5 µL external PCR reaction + 45 µL sterile water).
b. Set up external PCR reactions. Add components in order, to the bottom of a PCR tube on ice.
   10 µL sterile water
   5 µL diluted lysed worm solution (or 5 µL sterile water for negative control)
   5 µL external forward primer
   5 µL external reverse primer
   25 uL Master Mix (contains salts, dNTPs, Taq polymerase, loading dye and sterile water)
   -----
   50 µL total volume
c. Mix by gently by pipeting up and down about 12 times.
d. Put tube in thermocylcer and run PCR program:

Initial denaturation	92°C	3 min.
These steps loop 35 times:		
--denaturation	92°C	40 sec.
--annealing	52°C	40 sec
--extension	72°C	3 min.
Final extension	72°C	10 min.
4°C for ever		

(Page 11 has information on what happens during each step of the PCR reaction.)

2. **Perform internal PCR reactions for all samples.**
   a. Dilute external PCR reactions 1:10 (5 µL external PCR reaction + 45 µL sterile water).
   b. Follow steps b-d above, except use internal primers when setting up the PCR reactions in b.

F. Perform agarose gel electrophoresis of amplified DNA (Week 10).

In this procedure you will run out some of your internal nested PCR reactions on an agarose gel along with a DNA ladder. This will allow you to see if a deletion is present in the region that you amplified in the nested PCR for each of your samples. You should also be able to estimate the size of the deletion from the information on the gel.

Important safety precautions:

- We will add ethidium bromide to the gel staining solution to stain the DNA. Ethidium bromide is a powerful mutagen (it causes frameshifts). Wear gloves when preparing and handling the gel.
- The electrical current from the power supply is dangerous. The gel boxes have been designed so that current can run through them only when the cover is closed. Always turn off the power supply before opening the box.
- We will use UV light to see the ethidium bromide-stained DNA bands. UV can harm your eyes and you should never look directly at it. Be sure the glass cover is in place (in other situations, wear goggles)!

1. Pouring the gel.
Assemble the gel box as demonstrated by the staff. The side pieces should fit very tightly so that the box does not leak when molten agarose is poured in. Be sure the "comb," which will form the sample slots, is seated properly.

a. 0.8% agarose containing electrophoresis buffer TBE (Appendix A) will be available in a 70°C water bath. It is liquid at this temperature but will become solid as it cools in the gel box.
b. Pour in enough molten agarose to make a depth of 6 mm (~40 ml). When the gel has set it will look very cloudy (about 10-15 minutes). Add just enough TBE to be level with the top of the gel. Carefully remove the comb and end pieces. If there are any air bubbles in the sample slots, remove them with a pipette tip.

2. Loading the gel.

a. Thaw your amplified DNA. This can be done in a few minutes by holding it in your palm.
b. Load 15 µl of each of your samples (including controls) into consecutive gel slots. Keep track of what is loaded into each lane, following the lane assignments shown on the blackboard.
c. Load 7 µl of the molecular weight markers (they already include tracking dye) into a slot. These are 13 double stranded linear DNA fragments of known molecular weights of 10kB, 8kB, 6kB, 5kB, 4kB, 3kB, 2.5kB, 2kB, 1.5kB, 1kB, 0.75kB, 0.5kB and 0.25kB collected in a DNA ladder. The 1kB and 3kB fragments will have the brightest bands.

3. Running the gel.

a. DNA is negatively charged; hook up the leads so that the positive electrode is at the end opposite the sample slots.
b. Electrophorese the samples at 115 V until the fastest tracking dye has moved a little more than half way down the gel (this takes about 35 min). Turn off the power supply. If you let the electrophoresis go too long, it is possible that your smallest DNA fragments could migrate off the gel.

4. Staining the gel.

a. Wearing gloves, gently remove your gel from the casting tray and place it in a staining dish at the back of the room. The staining dishes contain 0.5µg/500ml ethidium bromide.
b. Let the gel stain for about ~30 minutes.

5. Photographing your gel.
(Wear gloves for this.) Transfer your gel to the UV light box and observe the bands. The UV light will not turn on until the safety cover is closed. The staff will help you take a Polaroid picture of your gel (f11, 1 second exposure).

References

Becker, WM, Kleinsmith LJ, Hardin J. (2006) World of the Cell. 6th edition. Pearson Benjamin Cummings Publisher.

Sonnhammer EL., Durbin R. (1997) Analysis of protein domain families in *Caenorhabditis elegans*. *Genomics* 46(2):200-16.

Culetto E, Sattelle DB. (2000) A role for *Caenorhabditis elegans* in understanding the function and interactions of human disease genes. *Hum Mol Genet*. 9:869-77.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 391:806-11.

Mello CC, Conte D Jr. (2004) Revealing the world of RNA interference. *Nature*. 431:338-42.

Timmons L, Fire A. (1998) Specific interference by ingested dsRNA. *Nature*. 395:854.

Appendix A

Recipes

PK Buffer
For 200 mL. . .
10 mL 1M KCl
9 mL 10% NP40 (Tergitol)
9 mL 10% Tween 20
2 mL Tris pH 8.3
0.5 mL 1M $MgCl_2$
40 mg proteinase K (Invitrogen, cat#: 25530-015)

LB (Luria-Bertani) Broth
For 1 L. . .
10 grams tryptone
5 grams of yeast extract
10 grams of NaCl
Autoclave 20 minutes.

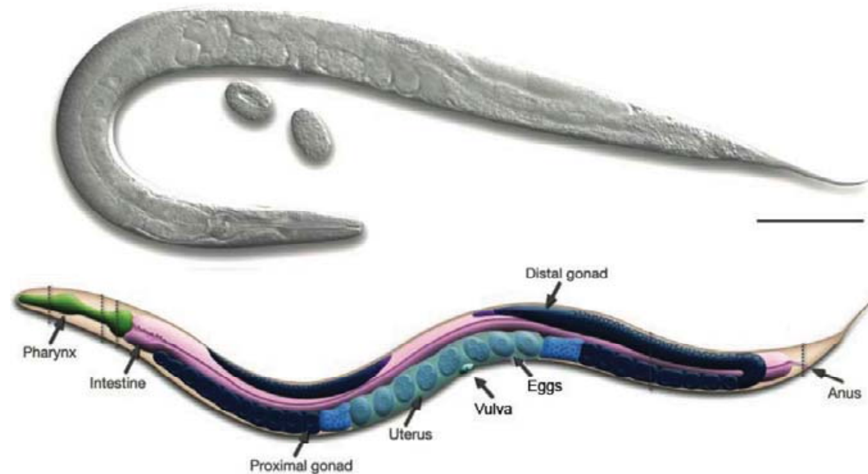
Tris-Borate-EDTA Electrophoresis Buffer, pH 8 (TBE)
[Tris is a commonly used buffer. Its structure is shown in the Notes at the end of the Enzyme Catalysis lab.]
0.09 M Tris-base
0.09 M boric acid
0.002 M EDTA

Feeding RNAi plates
For 1 L. . .
3 g NaCl
17 g agar
2.5 g Bacto-Peptone
1 mL 5 mg/mL cholesterol in ethanol
975 mL H2O
Autoclave 20 minutes, cool to 60C, then add:
1 mL 1 M $CaCl_2$
1 mL 1 M $MgSO_4$
25 mL 1 M KH_2PO_4, pH 6.0, filter sterilized
20 mL 10% lactose, filter sterilized

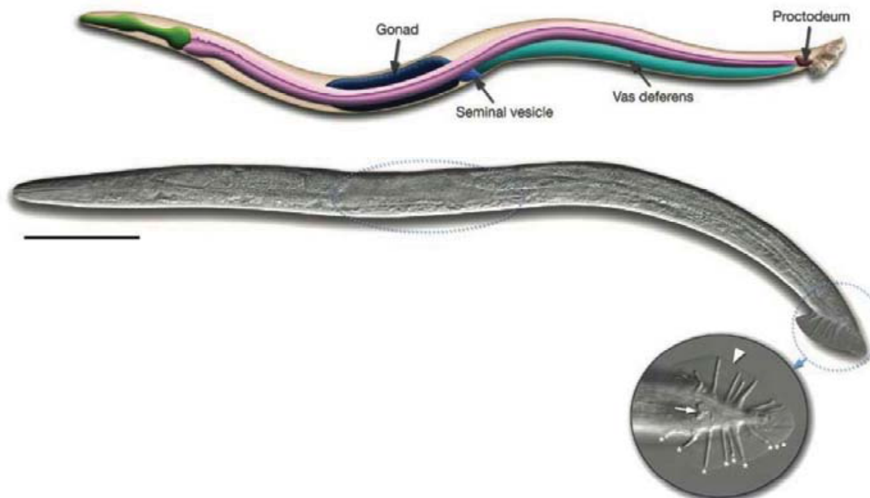
1 mL 25 mg/mL carbenicillin, filter sterilized
1 mL 50 mg/mL tetracycline, filter sterilized

Appendix B

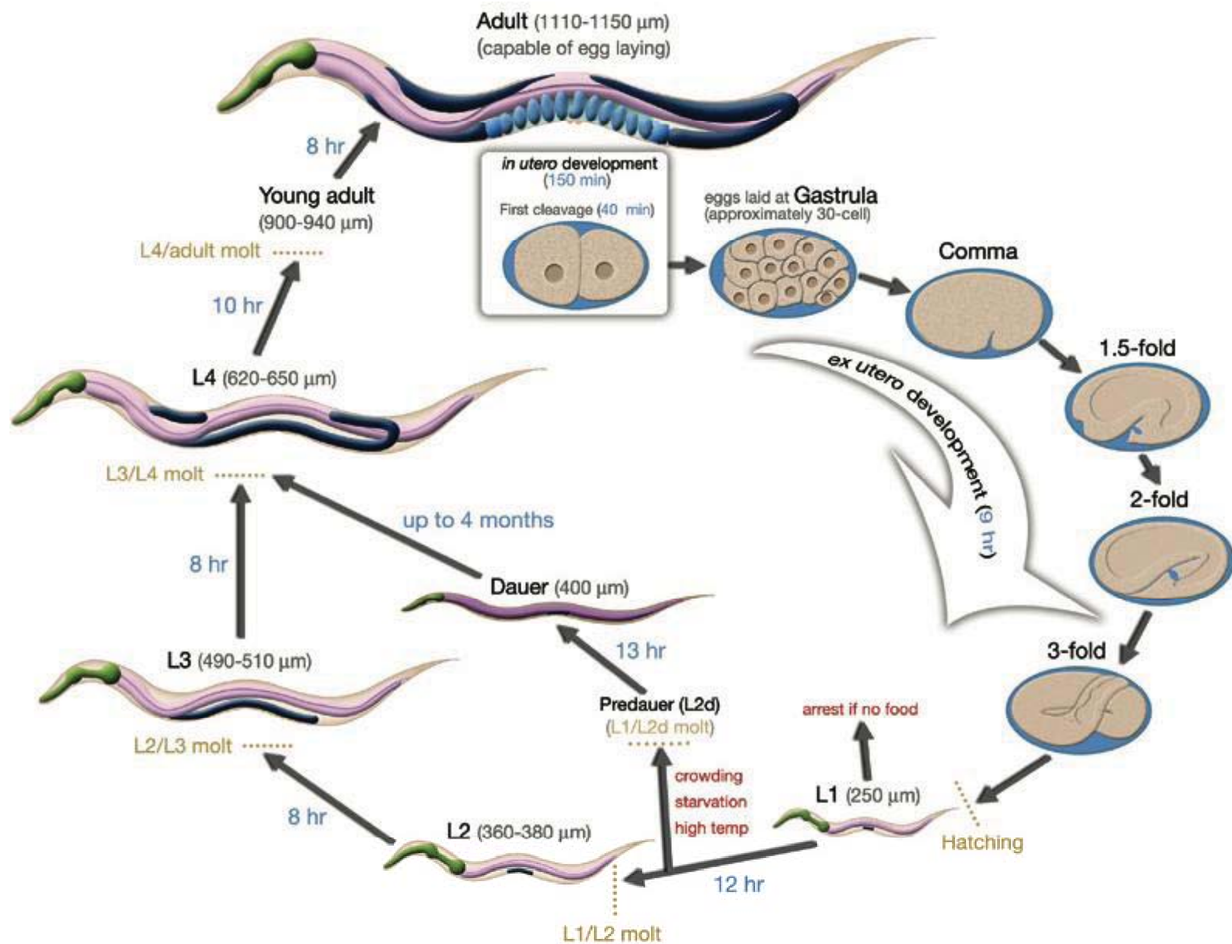
C. elegans Anatomy, Life Cycle, and List of Web Resources.



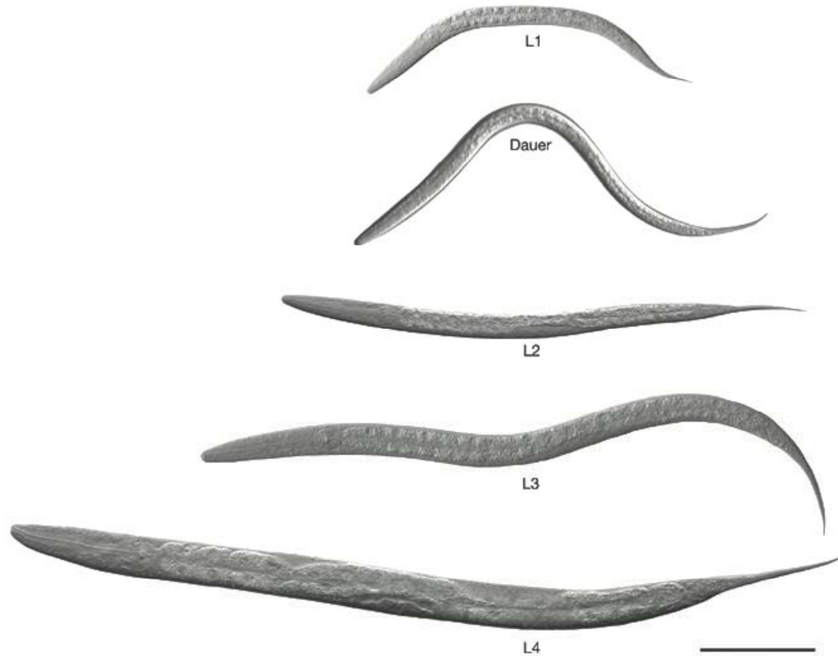
Supplemental Figure 1: *C. elegans* Anatomy. Top shows a DIC (differential interference contrast) image of an adult hemaphrodite with two eggs. The basic anatomical features of the worm are illustrated in the bottom picture. The pharynx has two bulbs that crush up bacteria. Nutrients are absorbed by the intestine. The worm has two gonad arms, and each is shaped like the letter "U". Oocytes are produced in the distal gonad and are fertilized in the proximal gonad. Eggs are laid out of the vulva. Some important features are not directly shown in the diagram including: the epidermis (which is the outer, skin-like layer of the worm), muscles (four longitutidinal muscles run along the length of the worm, two on the top and two on the bottom), and nerves. The scale bar is 0.1 mm. This has been modified from WormAtlas IntroFig1.



Supplemental Figure 2: *C. elegans* Male Anatomy. *C. elegans* males have a tail that is specialized for mating. Adult males are slightly smaller than adult hemaphrodites, and arise only rarely in *C. elegans* populations. Scale bar is 0.1 mm. This has been modified from WormAtlas IntroFig5.



Supplemental Figure 3: The *C. elegans* life cycle. Fertilization occurs at 0 min. Blue numbers along arrows indicate the length of time the animal spends at a certain stage. The length of the worm at each stage is indicated in micrometers. *C. elegans* goes through four larval stages (L1, L2, L3 and L4). If they are starved between the L1 and L2 stage then they will become dauer larva that can survive in a hibernation-like state for many months. <u>On your worm plates you will likely see eggs and worms that are at a mix of different stages.</u> This is from WormAtlas IntoFig6.



Supplemental Figure 4*:* *C. elegans* larval stages. DIC images of the different larval stages showing their size relative to each other. Bar is 0.1 mm. Adapted from WormAtlas IntroFig8B.

More information on *C. elegans* can be found at the following websites:

WormAtlas
Excellent resource on the anatomy of *C. elegans*.
http://www.wormatlas.org/

Worm Classroom
Has information on *C. elegans* presented at an introductory level and some nice movies of worms.
http://www.wormclassroom.org/

WormBook
Online reviews on topics involving the biology of *C. elegans.* They have a section on disease models and drug discovery.
http://www.wormbook.org/

WormBase
Includes information on the genome, and includes helpful information on what is know about each gene.
http://www.wormbase.org/

Authors: Elisabeth A. Cox and Theresa M. Grana
Some sections adapted from "What kinds of genes are in your Fritos? Detecting Genetically Modified Food by Polymerase Chain Reaction. "Janet Batzli and Diana Brandner, and

Phillips, A. R., Robertson, A. L., Batzli, J., Harris, M., Miller, S. (2008). Aligning goals, assessments, and activities: An approach to teaching PCR and gel electrophoresis. CBE-LSE. In press.

Robertson, A. L. and Phillips, A. R. (2008). Integrating PCR theory and bioinformatics into a research-oriented primer design exercise. CBE-LSE. In press.