Silencing Genomes

Laboratory 4

Inducing RNAi by Feeding

REAGENTS, SUPPLIES & EQUIPMENT CHECKLIST_____

This laboratory uses reagents that rely on procedures covered in Laboratory 2 (Culturing *C. elegans*) and Laboratory 3 (Culturing *E. coli*). Refer to these laboratories and the recipes section for details on reagent preparation.

Cultures and Media

☐ *E. coli* OP50 culture

☐ RNAi feeding strains *(dpy-10*, *bli-1*, and *unc-22)* seeded on NGM-lite/amp+IPTG plates (from Lab 3)

☐ Mutant worms (*dpy-10*, *bli-1*, *unc-22)* on NGM-lite plates (optional)

☐ OP50-seeded NGM-lite plates (from Lab 3)

☐ Starter plate of *rrf-3* worms

☐ Starter plate of *dpy-13* worms

☐ *dpy-13* RNAi feeding strain on NGM-lite/amp+IPTG plate

☐ *Lysis buffer (35 µL)

*Store on ice

Supplies and Equipment

☐ Binocular dissecting microscope

☐ Bunsen burner

☐ Permanent marker, black and red

☐ 95% ethanol, in 50- or 100-mL beaker

☐ Steel scalpel, spatula, or forceps

☐ Worm pick

☐ Container with cracked or crushed ice

☐ Container with liquid nitrogen or dry ice (or access to a -80°C freezer)

INTRODUCTION

Functional genetic studies typically rely on mutating a particular gene, then looking for physical or behavioral changes in the organism. Obviously, such experiments cannot be performed in humans, so biologists study physiology, cell biology, and many other biological functions in "model organisms." Although the microscopic worm *C. elegans* might seem an unlikely model for human biology, much of its cell biology, biochemistry, and development is similar to humans. Thus, it has become a key model system for studying gene function.

In recent years, the usefulness of the *C. elegans* model system has been dramatically enhanced because it is particularly suited to gene silencing by RNA interference (RNAi). RNAi is a mechanism that inhibits gene function when double-stranded RNA (dsRNA) molecules that correspond to part of a "target gene" are present in a cell. By deliberately introducing defined sequences of dsRNA, biologists can observe the physiological consequences of "silencing" virtually any gene in *C. elegans*, as well as many other plants and animals. Amazingly, this mechanism can be activated in *C. elegans* by simply feeding worms bacteria expressing dsRNA that corresponds to part of the gene to be silenced.

This experiment demonstrates how RNAi can be used to deduce the function of genes. Different cultures of wild-type worms are fed bacteria expressing dsRNA specific to a particular worm gene. Ingesting dsRNA initiates destruction of the mRNA of the target gene. An altered phenotype in the progeny of RNAi-treated worms indicates what happens when the normal function of this gene is lost. In accompanying bioinformatics exercises, students discover the function of the protein encoded by each silenced gene and explore the relatedness of genes in worms and humans.

In Part I, RNAi-sensitive worms *(rrf-3)* are chunked onto OP50-seeded NGM-lite plates. In Part II, *rrf-3* worms are transferred onto RNAi feeding strains *(dpy-10, bli-1, and unc-22)* on NGM-lite/amp+IPTG plates, as well as a control OP50-seeded NGM-lite plate.

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METHODS _____

I. Transfer *rrf-3 C. elegans* to OP50-seeded NGM-lite Plates

To feed worm strains, it is easier to transfer a chunk of worm-filled agar from a well-grown plate to a new plate rather than pick individual worms. The rrf-3 *strain of* C. elegans *is used because of an increased sensitivity to RNAi.*

Cultures	Supplies and Equipment
OP50-seeded NGM-lite plate (from Lab 3) Starter plate of *rrf-3* worms	Bunsen burner Permanent marker, black 95% ethanol, in 50- or 100-mL beaker Steel scalpel, spatula, or forceps

1. Use a *black* pen to label the bottom of your OP50-seeded plate with the date and "rrf-3."

2. Examine the plate under the microscope to verify the plate is free of bacterial or mold contamination. Obtain a new plate if your plate is contaminated.

3. Sterilize a metal spatula or forceps. Dip the end of the implement into the ethanol beaker, and *briefly* pass it through a Bunsen flame to ignite the alcohol. Allow alcohol to burn off *away from* the Bunsen flame; the implement will become too hot if left in the flame. (Sterilization prevents cross-contamination with different *C. elegans* strains and non-OP50 bacteria.)

CAUTION
Be extremely careful not to ignite the ethanol in the beaker. Do not panic if the ethanol is accidentally ignited. Cover the beaker with a Petri dish lid or other non-flammable cover to cut off oxygen and rapidly extinguish the fire.

4. Use the sterilized implement to cut a 1-cm (about 1/4–1/2 in.) square chunk of agar with worms from the *rrf-3* starter plate.

5. Transfer the chunk to your OP50-seeded plate, and place it face down in the agar. (This allows worms to quickly crawl into the bacteria on the new plate.)

6. Incubate the plate upside down at 20°C for 48 hours. Choose a place where the plate will not be disturbed. This will give the newly transferred worms time to grow.

II. Induce RNAi by Feeding

Cultures	Supplies and Equipment
Mutant worms (*dpy-10*, *bli-1*, *unc-22)* on OP50-seeded NGM-lite plates (optional) OP50 seeded NGM-lite plate (from Lab 3) RNAi feeding strains *(dpy-10, bli-1, and unc-22) on* NGM-lite/amp+IPTG plates (from Lab 3) *rrf-3* worms on NGM-lite plate (from Part I)	Binocular dissecting microscope Bunsen burner Permanent marker, black Worm pick

1. Use a *black* pen to label the *bottom* of your OP50-seeded plate with the date and "rrf-3."

2. Use a *black* pen to label the *bottom* of each of your RNAi feeding plates (*dpy-10, bli-1, unc-22)* with the date and "rrf-3."

3. Pick five L4-stage worms from your plate of *rrf-3* worms to the OP50-seeded plate labeled "rrf-3."

4. Confirm that the transferred worms are the correct stage and were not injured or killed during the picking process.

5. Identify any eggs or young larvae that may have been accidentally transferred. Pick them off the plate, and flame them in a Bunsen burner.

6. Use the same method to move five L4 *rrf-3* worms to each of the plates seeded with RNAi feeding strains (*dpy-10*, *bli-1*, and *unc-22).*

7. Incubate the plates upside down at 20°C. Choose a place where the plates will not be disturbed.

8. The day after transferring, check that your worms are still healthy. Note any dead worms, pick them off the plate, and flame them in a Bunsen burner.

9. On the third day after transferring, pick the remaining RNAi-treated adult worms off the plate, and flame them in a Bunsen burner. This will avoid confusion when scoring the progeny.

RESULTS & DISCUSSION _____

1. Starting on day two, carefully examine each plate.

   a. Have any eggs been laid? Have any eggs hatched? If so, are the worms at a larval or adult stage?

   b. Compare the progeny of the RNAi-treated worms to the progeny of the untreated *rrf-3* worms. Note any differences in morphology or behavior. At which developmental stage are the worms in which you observe these differences (L1, L2-3, L4, or adult)?

   c. Make a quantitative assessment of the RNAi knockdown on each plate, *looking only at the progeny of the original worms you transferred.* Once some of the 1st generation (F1) progeny of the RNAi-treated worms reach adulthood, look for any worms on the plate that have a phenotype. Do this before the 2nd generation (F2) reaches adulthood.

   d. Count the number of adult F1 worms that have a mutant phenotype and those that appear normal (wild-type). As you count each worm, remove it from the plate with a pick, and flame it in a Bunsen burner.

   e. Repeat this analysis again the following day for additional F1 worms that have reached adulthood.

   f. Calculate the percentage of F1 adult worms that were affected on each RNAi plate on each day. How effective was the RNAi treatment? Did the RNAi effect vary over time?

2. Given the phenotypes you observed, what can you deduce about the function of each gene that was knocked down?

3. NGM-lite plates on which the RNAi feeding strains are grown include ampicillin. Why is ampicillin added to these plates? How are the RNAi feeding strains of bacteria different from the OP50 strain, which are grown on plain NGM-lite plates?

BIOINFORMATICS _____

Information is encoded in the nucleotide sequence of DNA. Bioinformatics is the field that identifies biological information in DNA using computer-based tools. Some bioinformatics algorithms aid the identification of genes, promoters, and other functional elements of DNA. Other algorithms help determine the evolutionary relationship between DNA sequences.

Because of the large number of tools and DNA sequences available, experiments done *in silico* ("in silicon," or on the computer) now complement experiments done *in vitro* ("in glass," or in a test tube). This movement between biochemistry and computation is a key feature of modern biological research.

In Part I, you will use Wormbase, an online database for *C. elegans* research, to learn about one of the genes examined in the laboratory. Wormbase contains the entire *C. elegans* genome sequence and the locations of all predicted genes. An entry for each gene includes a summary of data from genetic, biochemical, and RNAi experiments. In Part II, you will use the Basic Local Alignment Search Tool (BLAST) to identify the human equivalent of the worm gene. This same approach can be repeated with the other genes examined in the laboratory.

I. Use Wormbase to Find Basic Information About the Gene

1. Open the internet site for Wormbase at
    http://www.wormbase.org/.

2. Enter "unc-22" in the text box adjacent to *Find: Any Gene* window, and then click the search button.

3. Look at the description of the gene. What does "unc" stand for? What sort of protein does the gene encode? What is the function of the gene? Follow any blue links to find additional information.

4. Scroll down to the *Location* section to examine a graphical representation of the structure of the *unc-22* gene on *C. elegans* chromosome IV, as well as a neighboring glucose transporter gene. Three *unc-22* transcripts are depicted, each running from right to left, or 5' to 3'. Exons are colored boxes, introns are thin black lines, and the 3' untranslated region is a black arrow. How can there be more than one transcript for this gene?

5. Scroll down to the *Function* section, which describes different alleles (mutations) of the *unc-22* gene. Each allele is given a different number. In this laboratory, you used *unc-22(e66),*

allele *e66* of the *unc-22* gene. Follow the blue link for a complete description of this allele.

II. Use BLAST to Find the Human Homolog of *unc-22*

1. Return to the *Identification* section at the top of the page, and find the *Gene model(s)* table. Click on the first entry in the *Nucleotides (coding/transcripts)* column, to retrieve the nucleotide sequence for the transcript "ZK617.1a.1"

2. The first entry is the spliced coding region (transcript) as it would be translated by the ribosome. (For simplicity's sake, the transcript is presented as DNA code, rather than RNA.) Exons are shaded in alternating yellow and orange (exon 1 is yellow, exon 2 is orange, exon 3 is yellow, etc.). The second entry is the unspliced gene. Exons are colored while introns, and 5' and 3' untranslated regions are white.

3. Use your mouse to highlight and copy the entire spliced sequence.

4. Initiate a BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) search.

   a. Open the Internet site of the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/.

   b. Click on *BLAST* in the top speed bar.

   c. Click on *Translated query vs. protein database (blastx).*

   d. Paste the *unc-22* coding sequence into the *Search* window.

   e. Delete any non-nucleotide characters from the window.

   f. In the *Options* section, choose "Homo sapiens" from the pull-down menu under "or select from All organisms."

   g. Click on *BLAST!*

   h. Click on *Format!* to send the query sequence to a server at the National Center for Biotechnology Information in Bethesda, Maryland. There, the BLAST algorithm will translate the coding sequence into amino acids and attempt to find a match with the amino acid sequences of human proteins currently stored in its database. This may take only a few seconds or several minutes if several searches are queued at the server.

i. Why would we do a BLAST search with amino acids rather than DNA sequence?

5. The results of the BLAST search are displayed in three ways on the output page:

   a. A graphical overview illustrates how significant matches (also known as "hits") align with the query sequence.

   b. A list of significant alignments with *Geneinfo Identifier* (*gi)* links.

   c. A detailed view of the translated *unc-22* sequence *(query)* aligned to the amino acid sequence of each search hit (*subject*). Amino acids that are identical to both sequences are listed in the center row, while biochemically similar amino acids are indicated by "+" signs.

6. Examine the hits from the BLAST search*.*

   a. In the list of *significant alignments*, notice the scores in the *E-value* column on the right. The *Expectation* or *E-value* is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the *E-value*, the higher the probability that the hit is related to the query. Longer queries generally yield lower *E-value*s.

   b. An alignment is considered significant if it has an *E-value* less than 0.1. The 0.0 values at the top of the list are obviously very significant, but consider that the value *6e-156* further down the list denotes 6×10^{-156} or a 6 preceded by 156 decimal places!

   c. Identify the hits with the lowest *E-values*. Hits with similar *E-values* are usually variants of the same sequence. What human protein is most related to *C. elegans* UNC-22? What *E-value* did it yield? Is it a significant match?

   d. Find a hit with a dark "G" next to the entry, and click on the "G". This link will open an Entrez Gene record for the human *unc-22* relative.

   e. From the *Display* drop-down menu at the top left, select *Full Report*. Scroll down, and notice that the report includes a graphic representation of transcripts for the gene, its chromosomal location, and a list of references. Does this gene have a similar function to *unc-22?* Is it important in human health?

   f. Follow any of the links to get more detailed information about the gene.

CONCEPTS AND METHODS _____

This laboratory can help students understand several important concepts of modern biology:
- The relationship between genotype, phenotype, and RNAi-induced phenotype.
- RNA transcription and complementary base pairing.
- Methods to study the function of genes.
- The connection between *in vitro* experimentation and *in silico* computation.

The laboratory uses several methods for modern biological research:
- Microscopy.
- Growth and manipulation of a model organism.
- Reverse genetics.

INSTRUCTOR PLANNING, PREPARATION, AND LAB FINE POINTS _____

The following table will help you to plan and integrate the different parts of the experiment.

Part	Day	Time	Activity
Pre-lab	−2	20 min.	**Pre-lab** Chunk *rrf-3* worms to OP50-seeded NGM-lite plates (from Lab 3) (one per student group).
I. Transfer *rrf-3 C. elegans* to OP50-seeded Plates	1	20 min.	**Pre-lab**: Set up student stations.
		20 min.	**Lab:** Chunk *rrf-3* worms to OP50-seeded NGM-lite plates (from Lab 3) (one per student group). Optional: Chunk mutant strains with similar phenotypes to those expected in the experiments (*bli-1, unc-22, and dpy-10* mutants) to OP50-seeded NGM-lite plates.
II. RNAi by Feeding	3	20 min.	**Pre-lab**: Set up student stations.
		60 min.	**Lab:** Pick L4 worms to the appropriate plates to induce RNAi.
	4	20 min.	**Pre-lab**: Set up student stations.
		20 min.	**Lab:** Examine plates and flame dead worms.
	6	20 min.	**Pre-lab**: Set up student stations.
		60 min.	**Lab:** Observe and score phenotypes.
	7	20 min.	**Pre-lab**: Set up student stations.
		60 min.	**Lab:** Observe and score phenotypes.
	8	20 min.	**Pre-lab**: Set up student stations.
		60 min.	**Lab:** Observe and score phenotypes. Discuss Results.

I. Transfer *rrf-3 C. elegans* to OP50-seeded Plates

An RNAi-sensitive strain (*rrf-3)* is used in this laboratory to increase the effectiveness of RNAi. This strain is temperature sensitive, so care should be taken to avoid exposing it to temperatures above 20°C.

Pre-lab Preparation

Two days before the lab, chunk *rrf-3* worms to OP50-seeded plates. Alternatively, have students prepare plates by completing Part I of Lab 2 with *rrf-3* worms.

Pre-lab Setup (per student station)

Binocular dissecting microscopes
Bunsen burner
OP50-seeded NGM-lite plate (from Lab 3)
Permanent marker, black
95% ethanol, in 50- or 100-mL beaker
Starter plate of *rrf-3* worms
Steel scalpel, spatula, or forceps

II. Induce RNAi by Feeding

To minimize the number of plates used and the time needed to pick worms, each student group can perform RNAi with one of the three RNAi feeding strains (*bli-1, unc-22,* and *dpy-10*). Groups can share plates to observe the results of RNAi for each gene.

Although optional, having mutant worm strains available as references makes it much easier to identify the RNAi phenotypes. Since each strain is homozygous for the mutation, every worm on the plate will exhibit the mutant phenotype. Penetrance – the percentage of worms expressing the knockdown phenotype – will vary among RNAi-treated worms.

Pre-lab Preparation

If desired, chunk mutant strains (*bli-1*, *unc-22*, and *dpy-10*) to NGM-lite plates for phenotypic comparison.

Pre-lab Setup (per student station)

Binocular dissecting microscopes
Bunsen burner
Mutant worms (*dpy-*10*, bli-1*, and *unc-22)* on OP50-seeded NGM-lite plates (optional)
OP50-seeded NGM-lite plate (from Lab 3)
Permanent marker, black
RNAi feeding strains *(dpy-10*, *bli-1*, and *unc-22)* on NGM-lite/amp+IPTG plates (from Lab 3)
rrf-3 worms on OP50-seeded NGM-lite plate (from Part I)
Worm pick

ANSWERS TO DISCUSSION QUESTIONS _____

1.a.   Have any eggs been laid? **By day 2, the worms should have laid embryos. If no embryos have been laid, the worms may be unhealthy or dead.** Have any eggs hatched? If so, are the worms at a larval or adult stage? **After one day, the eggs should hatch and larval worms should be present. These larvae will grow to adulthood in 3–4 days.**

1.b.   Compare the progeny of the RNAi-treated worms to the progeny of the untreated *rrf-3* worms. Note any differences in morphology or behavior. At which developmental stage are the worms in which you observed these differences (L1, L2-3, L4, or adult)? **The RNAi phenotypes typically are noticeable only in adult worms. The phenotype of RNAi-treated worms is similar to the worm strain carrying a mutation in the same gene: *bli-1* adults develop fluid-filled blisters in the cuticle, *unc-22* adults move slowly and twitch, and *dpy-10* adults have short bodies.**

1.f.   Calculate the percentage of F1 adult worms that were affected on each RNAi plate on each day. How effective was the RNAi treatment? Did the RNAi effect vary over time? **The RNAi treatment will vary both in the percentage of worms affected (penetrance) and the severity of the phenotype (expressivity). Penetrance and expressivity may be higher in F1 progeny that mature later. Because they hatched later, their mother had a longer exposure to the RNAi feeding bacteria and more time to develop a stronger RNAi response. The RNAi treatment should yield 70-100% blistered worms for *bli-1*, 60-100% uncoordinated worms for *unc-22* and 50-100% dumpy worms for *dpy-10*.**

2.   Given the phenotypes you observed, what can you deduce about the function of each gene that was knocked down? **bli-1 is required for development of the outer cuticle. *unc-22* is required for normal muscle development and function. *dpy-10* is required for normal body length.**

3.   NGM-lite plates on which the RNAi feeding strains are grown include ampicillin. Why is ampicillin added to these plates? How are the RNAi feeding strains of bacteria different from the OP50 strain, which are grown on plain NGM-lite plates? **Ampicillin is added to NGM-lite plates to select for bacteria carrying an ampicilin resistance gene. The feeding vector – the plasmid that expresses dsRNA in the RNAi feeding strains – possesses an ampicillin resistance gene.   OP50 is a "wild-type" strain of *E. coli* that has no plasmid vector and, thus, should be grown on plain NGM-lite plates.**

ANSWERS TO BIOINFORMATICS QUESTIONS _____

I.3.   Look at the description of the gene. What does "unc" stand for? What sort of protein does the gene encode? What is the function of the gene? **C. elegans genes that were first identified by a mutant phenotype are named with a three-letter abbreviation of that phenotype. "Unc" is an abbreviation for uncoordinated. *unc-22* encodes twitchin, a giant muscle protein. The UNC-22 protein is required for normal muscle development (morphology) and to regulate the actomyosin contraction-relaxation cycle.**

I.4. How can there be more than one transcript? **The different transcripts are the result of alternative splicing, in which gene exons are spliced together in different arrangements.**

II.4.i. Why would we do a BLAST search with amino acids rather than DNA sequence? **Key sequences of amino acids must be maintained to conserve essential protein function through evolutionary time. The degenerate nature of the genetic code – several different codons specifying the same amino acid – permits some DNA mutations to occur during evolution, provided the protein sequence is maintained. Since identical protein sequences could be encoded by different DNA sequences, a protein BLAST search provides a better assessment of conservation of gene function between different species.**

II.6.c. What human protein is most related to *C. elegans* UNC-22? What *E-value* did it yield? Is it a significant match? **UNC-22 is most related to titin (TTN). An *E-value* of 0.0 is highly significant, meaning that the two proteins are closely related – with little chance that the match is a chance occurrence.**

II.6.e. Does this gene have a similar function to *unc-22?* Is it important in human health? **Yes, they are both large muscle proteins required for muscle structure and contraction. Mutations in titin have been identified in some patients with hypertrophic cardiomyopathy, and antibodies against titin are produced in patients with the autoimmune disease scleroderma.**