

Creating an RNAi Feeding Strain

STUDENT LAB INSTRUCTIONS

INTRODUCTION

In 1990, a strange thing happened when Rich Jorgensen and his colleagues tried to produce petunias with dark purple flowers by introducing a purple pigment gene into the flower. They reasoned that extra copies of a purple pigment gene would produce extra pigment and help produce a darker colored flower. However, they were surprised to produce flowers that appeared variegated, spotty, or even completely white. What seemed to be happening was that the introduction of the extra copies of the pigment gene somehow triggered a mechanism that was inhibiting the function of both the introduced copies of the gene and the copies that naturally occur in the petunia.

In 1995, while working on the free-living nematode worm *Caenorhabditis elegans* (*C. elegans*), Susan Guo and Ken Kemphues were trying to shutdown expression of a gene called *par-1* by using RNA complementary to the messenger RNA (mRNA). The RNA used in this method was named antisense RNA, since its sequence is complementary to that of the "sense" strand of mRNA. This technique of reducing gene function had been conceived and developed in other systems as a tool for researchers to deduce gene function. Indeed, when Guo and Kemphues injected antisense RNA into *C. elegans* worms, the expression pattern of *par-1* was disrupted. Scientists reasoned that the antisense RNA was able to bind to the complementary sequence of the mRNA and somehow inhibit translation. However, this theory was soon refuted when a control experiment yielded unexpected results. Unaccountably, the injection of only "sense" RNA also appeared to reduce gene function. Obviously this contradicted the aforementioned theory since injected "sense" RNA was not complementary to mRNA, and thus could not bind to it.

In 1998, Andrew Fire and Craig Mello solved this mystery when they discovered that the technique scientists were using to produce single-stranded RNA also produced some double-stranded RNA (dsRNA). They deduced that the results of the "sense" and "antisense" RNA injection experiments could be explained if dsRNA was, in fact, the cause of the reduction in gene function observed in both experiments. They eventually discovered that the cells of *C. elegans* possess molecular machinery that uses dsRNA to interfere with gene function. Thus, the RNA interference technique was renamed RNA interference, or simply RNAi.

Scientists studying many different organisms, including petunia, soon discovered that this system of inactivating gene expression was a highly conserved mechanism. Furthermore, they found that the function of virtually all genes could be down-regulated through the RNAi mechanism simply by introducing dsRNA that corresponds to the DNA sequence of the gene. This was a revolutionary finding that gave scientists a powerful new tool to study gene function. Scientists could now quickly and easily "knockdown" the function of genes for which no mutation had been identified. Coupled with DNA sequence yielded from the successful genome sequencing projects in organisms such as worms, flies, and

several mammals, RNAi has allowed scientists to conduct genome-wide screens to systematically study the function of genes. These experiments have vastly increased our understanding of the gene function of many previously unknown or poorly understood genes.

Since its inception as a tool for scientists to explore the function of genes, RNAi researchers have begun to understand why the RNAi mechanism evolved in nature. RNAi is believed to defend cells from viruses and silence the activity of “jumping genes” called transposons. In addition to protecting the integrity of DNA, the RNAi system also plays a role in organizing chromosomal DNA, guiding DNA methylation, and silencing certain regions, such as heterochromatin. Finally, recent evidence even implicates the RNAi mechanism in controlling aspects of development in multicellular organisms, such as *C. elegans*.

In this protocol, students will be guided through the process of creating their own RNAi feeding vector to inactivate the function of a gene in the *C. elegans* worm. Students will use PCR to amplify part of the gene, ligate the resulting PCR product into the feeding vector, and transform the vector into *E. coli*. Once they have confirmed that the construct has been assembled correctly, they will use the vector to down-regulate gene function by feeding the genetically engineered *E. coli* to worms.

Students will also be given the bioinformatic tools that will allow them to design their own primers, allowing them to make their own feeding vectors.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.

Kamath et al (2001) *Genome Biology*, 2, 1-10.

Tabara, H., Grishok, A., and Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282, 430-431.

Tabara H., Sarkissian M., Kelly W.G., Fleenor J., Grishok A., Timmons L., Fire A., Mello C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123-32.

METHODS

I. ISOLATE GENOMIC DNA FROM WORMS

DNA will be isolated from wild-type (N2) worms that have been grown on NGM plates. Ensure that there will be enough N2 plates with young adults and L4 worms to do the experiment by chunking N2 worms to fresh OP50 plates two days before the experiment.

Reagents	Supplies and Equipment
Worm Lysis buffer with Proteinase K	Permanent marker
Wildtype worms growing on worm plates	Micropipet and tips (1-10 μ L)
	Microcentrifuge tube rack
	Microcentrifuge
	Container with liquid Nitrogen or dry ice, or access to a -80°C freezer.
	Thermal cycler or hot water bath (65°C/90°C)
	Worm pick
	Dissection microscope
	PCR tubes (0.2 or 0.5 μ L)
	Microcentrifuge adaptors for 0.2 mL or 0.5 mL PCR tubes
	Bunsen burner

Make sure you have your microscope adjusted to your eyes. If you are having trouble seeing the worms, ask your teacher to help you.

It is critical to flame the pick in between worm manipulations to ensure that bacterial cross-contamination does not occur.

CAUTION! When using an open flame, use appropriate precautions. Make sure any loose clothing is secured, and tie long hair back. Do not lean over the flame.

The sample tubes are “nested” within sequentially larger tubes. 0.2 ml within 0.5 ml within 1.5 ml. Remove caps from the tubes used as adapters.

If a -80°C freezer is available, worms frozen in the lysis buffer are stable indefinitely. If the worms are frozen for a prolonged time, addition of extra another aliquot of proteinase K may be advisable before beginning step 6.

One of the main ingredients in lysis buffer is an enzyme called proteinase K. Proteinase K is a non-specific protease, an enzyme that cleaves the peptide bonds of proteins. It will help separate the nucleic acids from potential protein contaminants.

Worm DNA can be stored at 4°C for several weeks or at -20°C indefinitely.

1. Obtain a PCR tube. Label it with your group number.
2. Place 10 μ L of worm lysis buffer into each tube.
3. Using a dissecting microscope and a worm pick, pick 3-5 medium-sized wild-type worms and put them into the lysis buffer in the tube. Focus the microscope on the worms you have just placed in the lysis buffer. Verify that you can see at least 3 worms writhing in the buffer. The writhing action ensures that they are still alive and were not damaged in the picking process.
4. Optional: Place the PCR tube into an adapter tube (use a 1.5 mL eppendorf tube for 0.5 μ L PCR tubes and both a 1.5 mL and a 0.5 mL tube for 0.2 mL PCR tubes), so your PCR tube will not become lodged in the centrifuge. Briefly spin the worms to the bottom of the tube for 5 seconds.
5. Freeze tubes in liquid Nitrogen, on dry ice, or in a -80°C freezer for at least 10 minutes. Freezing and then thawing the worms will help break them open.
6. To lyse the worms, heat the tubes to 65°C for 60 to 90 minutes in a PCR machine or hot water bath.
7. After the lysis reaction is complete, inactivate the Proteinase K by heating the tubes to 95°C for 15 minutes in a PCR machine or water bath.

II. AMPLIFY DNA BY PCR

To amplify the DNA by PCR, you will need PCR reagents, the PCR primers for the experiment and access to a thermal cycler.

Reagents (at each student station)	Supplies and Equipment
*Worm DNA (from Part I)	Permanent marker
* <i>dpy-10</i> primer/loading dye mix, 30 μ L	Micropipet and tips (1-10 μ L and 10-100 μ L)
Ready-To-Go™ PCR Beads	Microcentrifuge tube rack
Mineral oil, 5 mL (if necessary for the thermal cycler)	Container with cracked or crushed ice
	Thermal cycler
*Store on ice	

If your thermal cycler does not have a heated lid: Prior to thermal cycling, you must add a drop of mineral oil on top of the reaction mixture. Be careful not to touch the dropper tip to the tube or cell suspension, or the oil will be contaminated with your sample.

1. Prepare PCR reactions:
 - a. Obtain a PCR tube containing a Ready-To-Go™ PCR Bead. Label with your group number.
 - b. Use a micropipet with a fresh tip to add 22.5 μ L of the *dpy-10* primer/loading dye mix to each tube.
 - c. Use a micropipet with a fresh tip to add 2.5 μ L of worm DNA (made in Part I) to the reaction tube.
2. Store samples on ice until you are ready to begin thermal cycling.
3. Program the thermal cycler for 30 cycles with the following profile. The program may be linked to a 4°C hold program after the 30 cycles are completed.

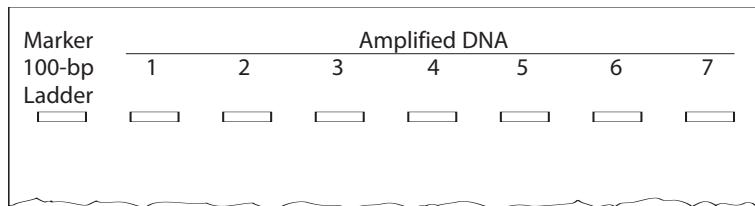
Denaturing step:	94°C	30 seconds
Annealing step:	55°C	30 seconds
Extending step:	72°C	60 seconds
4. After cycling is complete, store the amplified DNA at –20°C until you are ready to continue with Part III.

III. ANALYZE PCR PRODUCT BY GEL ELECTROPHORESIS

Reagents (at each student station)	Supplies and Equipment
* Amplified <i>dpy-10</i> DNA (from Part II)	Micropipet and tips (1-10 μ L)
1% agarose in 1X TBE, 50 mL	Microcentrifuge tube rack
1X TBE, 300 mL	1.5 mL microcentrifuge tube
Shared Reagents	Gel casting trays and combs
*100-bp ladder marker, 5 μ L (per team)	Gel electrophoresis chamber
Ethidium bromide (1 μ g/mL), 250 ml	Power supply
or	Staining trays
<i>Carolina</i> BLU™ Gel & Buffer Stain, 7 mL	Latex gloves
<i>Carolina</i> BLU™ Final Stain, 250 mL	UV transilluminator
*Store on ice	Digital or instant camera (optional)
	Water bath (60°C)
	Container with cracked or crushed ice
	Masking tape (if necessary)

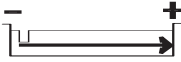
1. The exact procedure for casting an agarose gel will vary according to manufacturer. The following instructions refer to gel boxes which do not include a specific casting apparatus.
2. If necessary, seal the ends of the gel-casting tray with masking tape and place combs into the gel casting tray.
3. Pour 1% agarose solution into the tray to a depth that covers about 1/3 the height of the open teeth of the combs.
4. Allow the gel to solidify completely. This takes approximately 20 minutes.
5. Place the gel into the electrophoresis chamber, and carefully remove the comb.
6. Load 5 μ L of 0.1 μ g μ L 100-bp ladder into one of the wells.
7. Use a micropipet with a fresh tip to add 5 μ L of the amplified DNA (from part II) into different wells, according to the following scheme:

If you used mineral oil during PCR, be sure to pierce your pipette tip through the layer of mineral oil to withdraw the PCR products, leaving the mineral oil behind in the original tube.



Adding excessive running buffer will slow DNA movement in the gel.

8. Add enough running buffer (1X TBE buffer) to cover the surface of the gel.
9. Run the gels at 130 V for approximately 30 minutes. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.
10. Stain the gel using ethidium bromide or *Carolina*BLU™:



- a. For ethidium bromide, stain the gel 10-15 minutes. Once staining is complete, decant leftover staining solution back into a storage container for reuse. Briefly soak the gel in tap water to destain the gel. Rinsing or soaking the gel in water for a few minutes will remove excess ethidium bromide from the gel. *Use gloves when handling the ethidium bromide solution, a stained gel, or anything that has ethidium bromide on it. Ethidium bromide is a known mutagen and care should be taken when using and disposing of it.*
 - b. For CarolinaBLU™ staining, follow directions in the Instructor Planning section of the kit.
11. View ethidium bromide-stained gels using a UV transilluminator, and photograph the gels with a digital or instant camera.
 12. Confirm that the PCR reaction gives the expected band size with no non-specific contaminants.

IV. LIGATE PCR PRODUCT TO VECTOR

The PCR product now will be inserted into the RNAi feeding vector using ligase. Ligase and ligase buffer are sensitive to heat and should be stored carefully on ice when not in use.

Reagents	Supplies and Equipment
Amplified DNA (from Part II) *	Micropipet and tips (1-10 μ L)
Ligase mix (10 μ L)*	Microcentrifuge tube rack
	Microcentrifuge tubes
	Microcentrifuge adapters for 0.2 mL or 0.5 mL PCR tubes (optional)
*Store on ice	Microcentrifuge (optional)
	Container with cracked or crushed ice
	Permanent marker

The ligase mix includes the enzyme, ligase, ligase buffer including ATP and the RNAi feeding vector DNA that will be ligated to the PCR fragment.

1. Label an empty 1.5 mL tube with your group number and "L."
2. Use a micropipet with a fresh tip to add 10 μ L of amplified DNA to the tube marked "L."
3. Use a micropipet with a fresh tip to add 10 μ L of ligase mix to the tube marked "L."
4. Tap the tube with a finger to mix or pulse spin in a microcentrifuge using an adapter.
5. Incubate tubes at room temperature for at least two hours.
6. At this point, the ligation can be stored at -20°C until you are ready to proceed to Part VI.

V. PREPARE COMPETENT CELLS

The strain of E. coli used to induce RNAi in worms transforms inefficiently and is inappropriate for the production of high quality DNA. In order to ensure that transformation with the ligated DNA product will work efficiently, making highly competent E. coli cells is necessary.

Reagents	Supplies and Equipment
CaCl ₂ (50mM) (15mL)	Micropipet and tips (100-1000 μL)
Mid-log DH5α cells (two 10-mL cultures)	Container with cracked or crushed ice
	Permanent marker
	Waste Beaker
	Test tube rack
	Clinical centrifuge (2000-4000 rpm)
	Shaking incubator (37°C)
	Spectrophotometer (optional)
	Sterile pipettes (5 or 10 mL) and pipette aid or bulb
	Paper towels

This entire experiment *must be performed under sterile conditions.*

1. Place sterile tube of CaCl₂ solution on ice.
2. Obtain two 15-mL tubes each with 10 mL of mid-log cells and label with your name or group number.
3. *Securely close caps* and place both tubes of cells in a *balanced* configuration in the rotor of the clinical centrifuge. Centrifuge at 3000 rpm for 10 minutes to pellet cells on the bottom-side of the culture tubes.
4. Pour off the supernatant from each tube into a waste beaker for later disinfection. *Be careful not to disturb the cell pellet.*
5. Use a 5- or 10- mL pipette to add 5 mL of ice-cold CaCl₂ solution to each culture tube.
6. Immediately finger vortex to resuspend the pelleted cells in each tube by vigorously hitting the bottom of the tube while holding the top of the tube securely with the other hand. Do this until no visible clumps of cells remain in the tube. Alternatively, the cells can be resuspended by using a pipette. Using a sterile tip, break up cell clumps by repeatedly pipetting to the first stop.
7. Return both tubes to ice, and incubate for 20 minutes.
8. Following incubation, re-spin the cells in a clinical centrifuge for 5 minutes at 2000-4000 rpm. Following this spin, the cell pellet on the bottom of the tube will appear more spread out due to the CaCl₂ treatment.
9. Pour off the CaCl₂ supernatant from each tube into a waste beaker. Be careful not to disturb the pellet.
10. Use a 100-1000 μL micropipettor to add 1000 μL of CaCl₂ to each

tube.

11. Close caps tightly and immediately finger vortex to resuspend pelleted cells in each tube. See step 6.

Store cells in a beaker of ice in the refrigerator (~0°C) until ready to use. Incubation at 0°C for up to 24 hours can increase the competency of cells five- to ten-fold.

VI. TRANSFORM COMPETENT *E. coli* CELLS WITH LIGATED DNA

The ligation procedure creates recombinant DNA molecules that confer ampicillin resistance to the bacteria after transformation. Once the cells from Part V are competent, the following procedure can be used to move the DNA inside the cells.

Reagents	Supplies and Equipment
Ligated DNA (from part IV)*	Micropipet and tips (1-10 µL)
L4440 <i>dpy-10</i> DNA (control, 4 µL)*	Micropipet and tips (10-100 µL)
Competent cells (from part V)*	Microcentrifuge tubes
2 LB and 2 LB+AMP plates	Microcentrifuge tube rack
LB Broth (2 mL)	Container with cracked or crushed ice
	Permanent marker
	Masking tape
	Water baths (37°C and 42°C)
	37°C incubator (or shaking 37°C incubator, optional)
	Beaker of 95% ethanol and cell spreader (or spreading beads)
*Store on ice	

This entire experiment *must be performed under sterile conditions.*

1. Use a permanent marker to label two tubes with your group number. Label one tube "L," for ligated DNA, and one tube "C," for control.
2. Use a 100-1000 µL micropipettor and a *sterile tip* to add 200 µL of competent cells to each tube.
3. Place the tubes on ice.
4. Use a micropipettor with a fresh tip to add 10 µL of ligated DNA from Part IV to the tube labelled "L."
5. Use a micropipettor with a fresh tip to add 3 µL of L4440 *dpy-10* DNA to the tube labelled "C."
6. Close the caps, and tap the tubes to finger mix them.
7. Place the tubes on ice for at least 20 minutes.
8. While the cells are incubating on ice, use a permanent marker to label two LB plates and two LB/amp plates with your name (or group number) and the date.
 - a. Label one LB/amp plate "+." This is the experimental plate.
 - b. Label the other LB/amp plate "-." This is a negative control.

Label the bottoms of the plate where the agar is located. This will prevent confusion if the lid and bottom of the petri dish become separated.

It is critical that the cells receive a sharp and distinct shock. Removing the tubes from ice and then bringing the tubes to the water bath will cause this step to fail.

If the tubes are not immediately moved from the ice to the water bath, heat shock will not occur.

- c. Label one LB plate "+." This is a positive control.
 - d. Label one LB plate "-." This is a positive control.
9. Following the 20 minute incubation on ice, heat-shock the cells in both tubes.
 - a. Carry the ice beaker to the water bath. Remove the tubes from ice, and *immediately* immerse them in a 42°C water bath for 90 seconds.
 - b. Following the 90 second heat shock, immediately return both tubes to ice for at least 1 additional minute.
 - c. Leave the tubes at room temperature until you are ready to proceed to step 10.
 10. Use a micropipettor with a fresh sterile tip to add 800 µL of LB broth to each tube. Gently tap the tubes with your finger to mix.
 11. Allow the cells to recover by incubating the tubes in a 37°C water bath or incubator with moderate agitation for 20 to 30 minutes.
 12. Use a micropipettor with a *fresh sterile tip* to add 100 µL of cell suspension from the tube labeled "L" to the two plates labeled "L."
 13. Use a micropipettor with a *fresh sterile tip* to add 100 µL of cell suspension from the tube labeled "C" to the two plates labeled "C."
 14. Spread the aliquoted cells over the surface of the plates either using a cell spreader or sterile glass spreading beads. Remember to use sterile technique to avoid cross-contamination.
 15. Stack the plates (so they are all facing down), and tape them together to keep all the plates from your group together.
 16. Place the plates upside down in a 37°C incubator for 15-24 hours.
 17. Following the incubation, store the plates at 4°C to arrest *E. coli* growth and to slow the growth of any contaminating microbes until you are ready to proceed to Part VII.

VII. PERFORM SINGLE COLONY PCR AND INOCULATE OVERNIGHT CULTURES

Reagents	Supplies and Equipment
Plates with transformants on LB plus ampicillin plate "L" (from part IV)	Permanent marker
*T7 primer/loading dye mix, 120 μ L	Micropipet and tips (1-10 μ L and 1-100 μ L)
LB plus ampicillin in 15 mL snap cap tubes (5 mL) (4)	Microcentrifuge tube rack
Ready-To-Go™ PCR Beads (4 per group)	Test tube rack
Mineral oil, 5 mL (if necessary for the thermal cycler)	Container with cracked or crushed ice
	Thermal cycler
	Shaking incubator (37°C)
*Store on ice	

Although the ligation reaction should be efficient and specific, it is possible that some transformants will not possess the intended vector. To ensure that the desired product has been obtained, PCR will be used to determine the size of the fragment inserted into the vector in transformed cells. T7 primers that bind to the regions flanking the insertion site will be used in this verification process.

1. Prepare PCR reactions and inoculate culture tubes:
 - a. Obtain 4 PCR tubes containing Ready-To-Go™ PCR Beads. Number the tubes "1" through "4" and label them with your group number.
 - b. Number four 15 mL snap cap tubes containing LB plus ampicillin "1" through "4" and label them with your group number.
 - c. Use a micropipet with a fresh tip to add 25 μ L of the T7 primer/loading dye mix to each tube.
 - d. If the transformation in Step VI worked well, the LB amp plate marked "L" should possess many bacterial colonies. Circle the location of four colonies on the bottom of the plate. Number the circled colonies "1" through "4."
 - e. Use a micropipet with a fresh tip to pick up a part of the cell mass from colony 1. Aim to have the cell mass at the end of the tip. Immediately dip the end of the tip into the PCR tube labeled 1. Twirl the micropipet so that part (but not all) of the cell mass is released into the primer/loading dye mix.
 - f. Immediately after dipping the tip into the PCR tube, inoculate an overnight culture with the rest of the cell mass by ejecting the tip into the 15-ml tube labeled 1. Be sure to use sterile technique.
 - g. Repeat steps e and f for the remaining colonies, picking a bit of the cell mass for each colony to their respective PCR and 15-ml tubes.
2. Store the PCR tubes on ice until you are ready to begin thermal cycling.
3. Program the thermal cycler for 30 cycles with the following profile.

The program may be linked to a 4°C hold program after the 30 cycles are completed.

Denaturing step: 94°C 30 seconds

Annealing step: 52°C 30 seconds

Extension step: 72°C 90 seconds

4. Once PCR is complete, store the amplified DNA at -20°C until you are ready to continue with Part VIII.
5. Place the culture tubes in a 37°C incubator and shake for 12-16 hours (a typical incubation would last overnight).

The overnight cultures can be stored at 4°C for 2 to 3 days before proceeding to part VIII.

VIII. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

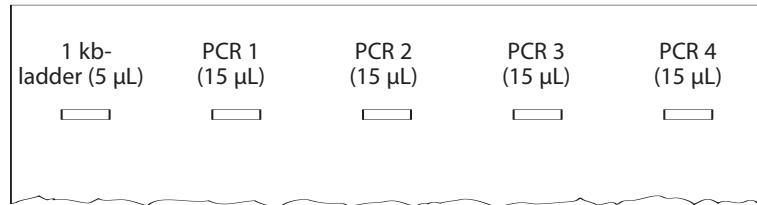
The size of the PCR product generated in part VII can be estimated to determine if the correct insert has been inserted into the vector. To determine the size of the PCR products, the PCR products will be run on an agarose gel and be compared to a size standard.

Reagents	Supplies and Equipment
Amplified DNA (from part VII)*	Micropipet and tips (1-10 µL)
1% agarose in 1X TBE, 50 mL	Micropipet and tips (10-100 µL)
1X TBE, 300 mL	Microcentrifuge tube rack
Shared Reagents	1.5 mL microcentrifuge tube
*1-kb ladder marker, 5 µL (per team)	Gel casting trays and combs
Ethidium bromide (1 µg/mL), 250 mL	Gel electrophoresis chamber
or	Power supply
CarolinaBLU™ Gel & Buffer Stain, 7 mL	Staining trays
CarolinaBLU™ Final Stain, 250 mL	Latex gloves
*Store on ice	UV transilluminator
	Digital or instant camera (optional)
	Water bath (55°C)
	Container with cracked or crushed ice
	Masking tape (if necessary)

1. The exact procedure for casting an agarose gel will vary according to manufacturer. The following instructions refer to gel boxes which do not include a specific casting apparatus.
2. If necessary, seal the ends of the gel-casting tray with masking tape and place a comb into the gel casting tray.
3. Pour a 1% agarose TBE solution to a depth that covers about 1/3 the height of the open teeth of the combs.
4. Allow the gel to solidify completely. This takes approximately 20 minutes.
5. Place the gel into the electrophoresis chamber, and carefully remove the comb.
6. Load 5 µL of a 0.1 µg/µL 1kb ladder solution into the first well.
7. Use a micropipet with a fresh tip to add 15 µL of the amplified DNA

If you used mineral oil during PCR, be sure to pierce your pipette tip through the layer of mineral oil to withdraw the PCR products, leaving the mineral oil behind in the original tube.

(from part VII) into different wells, according to the following scheme:



Adding excessive running buffer will slow DNA movement in the gel.

8. Add enough running buffer (1X TBE buffer) to cover the surface of the gel.
9. Run the gels at 130 V for approximately 30 minutes. Adequate separation has occurred when the cresol red dye front has moved at least 50 mm from the wells.
10. Stain the gel using either ethidium bromide or *CarolinaBLU*TM.
 - a. For ethidium bromide, stain the gel 10-15 minutes. Once staining is complete, decant leftover staining solution back into a storage container for reuse. Briefly soak the gel in tap water to destain the gel. Rinsing or soaking the gel in water for a few minutes will remove excess ethidium bromide from the gel. *Use gloves when handling the ethidium bromide solution, a stained gel, or anything that has ethidium bromide on it. Ethidium bromide is a known mutagen and care should be taken when using and disposing of it.*
 - b. For *CarolinaBLU*TM staining, follow directions in the Instructor Planning section.
11. View ethidium bromide-stained gels using a UV transilluminator. Photograph the gels with a digital or instant camera.
12. Determine the size of the PCR fragments by comparing them to the DNA bands of known size in the 1-kb ladder lane. Identify which transformants contain the correct insert.

IX. PERFORM PLASMID MINIPREP

Reagents	Supplies and Equipment
<i>E. coli</i> cultures of transformants from Part VII	Micropipet and tips (10-100 μ L) Micropipet and tips (100-1000 μ L)
Ethanol (95% or 100%), 1 mL	Waste beaker (2)
Glucose/Tris/EDTA (GTE), 500 μ L	Microcentrifuge tubes (8) Microcentrifuge tube rack
Isopropanol, 500 μ L	Container with cracked or crushed ice
Potassium acetate/acetic acid (KOAc), 800 μ L*	Bleach or disinfectant
SDS/sodium hydroxide (SDS/NaOH), 1 mL	Paper towel
Tris/EDTA (TE), 75 μ L	Microcentrifuge
	Permanent marker
*store on ice	

1. Obtain the *E. coli* overnight culture (from Part VII) of one transformant that contains the correct-sized insert (as determined in part VIII). Shake the culture to resuspend the *E. coli* cells that have settled to the bottom of the tube.
2. Label a 1.5 mL microcentrifuge tube with your group number and the number corresponding to the culture tube. For instance, if the culture tube is labeled "2," label the microcentrifuge tube "2" as well.
3. Use a micropipettor and fresh pipette tip to transfer 1000 μ L from the overnight suspension tube to the newly labeled microcentrifuge tube.
4. Close the cap and place the tube in a *balanced* configuration in the microcentrifuge. Spin at maximum speed for 1 minute to pellet cells.
5. Decant the supernatant from the tube into a waste beaker for later disinfection. Alternatively, use a micropipettor to remove the supernatant. *However, be sure not to disturb the pellet.* Invert the tube, and tap gently on the surface of a clean paper towel to drain thoroughly.
6. Add 100 μ L of ice-cold GTE solution to the tube. Resuspend the bacterial pellet in this solution by pipetting the solution in and out of the tip several times. Hold the tube up to the light to verify that the suspension is homogeneous and no visible clumps of cells remain.
7. Add 200 μ L of SDS/NaOH solution to the tube. Close the cap, and mix the solutions by rapidly inverting the tube five times. A white precipitate will immediately appear.
8. Put the tube on ice for 5 minutes. The suspension will become relatively clear.
9. Add 150 μ L of *ice-cold* KOAc solution to the tube. Close the cap, and mix the solutions by rapidly inverting five times. A white precipitate will immediately appear.
10. Leave the tube on ice for 5 minutes.
11. Place the tube in a *balanced* configuration in the microcentrifuge.

Avoid pipetting the precipitate and wipe off any precipitate clinging to the outside of tip prior to expelling the supernatant. Discard the old tubes which contain the precipitate.

Isopropanol preferentially precipitates nucleic acids; however, proteins remaining in solution also begin to precipitate with time.

After adding the isopropanol, proceed quickly. Leaving the supernatant in isopropanol for longer than 2 minutes before spinning will increase salt precipitation.

Align tubes in the rotor so that the cap hinges point outward. This will ensure that the nucleic acids pellet toward the bottom of the tube in the area underneath the hinge during centrifugation. The DNA pellet is sometimes clear and difficult to see, but you can assume it is there.

If the pellet is drawn into the tip, transfer the supernatant to another 1.5 mL tube, recentrifuge, and remove the supernatant again.

All ethanol must be evaporated before proceeding. Hold each tube up to the light to check that no ethanol droplets remain. If ethanol is still evaporating, an alcohol odor can be detected by sniffing the mouth of the tube.

A pellet of DNA with little salt contamination is sometimes difficult to see. As noted above, wash the area you expect the pellet to be located as DNA regardless of whether or not you see a pellet.

Spin the tube for 5 minutes at maximum speed to pellet the white precipitate.

12. Transfer 400 μL of the supernatant from the tube into a clean 1.5 mL tube also labeled with your group number.
13. Add 400 μL of isopropanol to the tube of supernatant. Close the cap, and mix the supernatant vigorously by rapidly inverting the tubes five times.
14. Place the tube in a *balanced* configuration in the microcentrifuge. Spin the tube for 5 minutes at maximum speed to pellet the nucleic acids.
15. Carefully pour off the supernatant from the tube. Remove any remaining liquid with a medium-sized pipetter set at 100 μL .
16. Add 200 μL of 95% or 100% ethanol to the tube, and close the cap. Flick the tube several times to wash the pellet.
17. Place the tube in a *balanced* configuration in the microcentrifuge, and spin it for 2-3 minutes at maximum speed.
18. Carefully pour off the supernatant from the tube. Then, completely remove the remaining liquid with a medium-sized pipetter set at 100 μL .
19. Air-dry the pellet for 10 minutes to evaporate the remaining ethanol.
20. Add 15 μL of TE to the tube. Resuspend the pellet by repeatedly rinsing it with the same 15 μL of TE. Be sure to rinse the side of tube several times, concentrating on the area where the pellet should have formed during centrifugation (beneath the cap hinge). Ensure that the all DNA is dissolved by verifying that no particles remain in the tip or on the side of the tube.
21. The miniprep DNA can be stored at 4°C short-term or 20°C long-term until you are ready to proceed to Part X.

X. TRANSFORM THE VECTOR INTO AN RNAi FEEDING *E. coli* STRAIN

Once the desired product is confirmed, the vector must be introduced into HT115(DE3). This strain of *E. coli* has an inducible gene that codes for T7 DNA polymerase. T7 DNA polymerase uses the T7 promoters located in the vector to transcribe RNA from both strands of the insert. HT115(DE3) also lacks an RNase that would digest the dsRNA produced by the vector. For this experiment, you will need to streak and incubate a plate of HT115(DE3) overnight at 37°C the day before you plan to do the transformation.

Reagents	Supplies and Equipment
Miniprep DNA (from part IX)*	Micropipet and tips (1-100 µL)
CaCl ₂ (50 mM) (600µL)	Micropipet and tips (100-1000 µL)
"starter plates" of HT115(DE3) <i>E. coli</i> grown overnight on LB plates	Microcentrifuge tube rack
LB Broth (600 µL)	Container with cracked or crushed ice
2 LB and 2 LB+AMP plates	Beaker of 95% ethanol and cell spreader (or spreading beads)
	Innoculating loop
	Bunsen Burner
	Test tube rack
	Permanent marker
	Culture tubes (two 15 mL)
	Water baths (37°C and 42°C)
	37°C incubator (or shaking 37°C incubator, optional)
*store on ice	Masking tape

1. Use a permanent marker to label one sterile 15 mL tube "+" plasmid. Label a second 15 mL tube "-" plasmid. DNA will be added to the "+" tube but not to the "-" tube.
2. Use a 100-1000 µL micropipettor and sterile tip to add 250 µL of CaCl₂ solution to each tube.
3. Place both tubes on ice.
4. Use a sterile inoculating loop to transfer one or two large (3 mm) colonies from the starter plate to the "+" plasmid tube:
 - a. Sterilize the loop in a Bunsen burner flame until it glows red hot. Then, pass the lower half of the shaft through the flame.
 - b. Stab the loop several times at the edge of the agar plate to cool.
 - c. Scrape a visible mass of cells by pick a couple of large colonies. Be careful not to transfer any agar because impurities in the agar can inhibit transformation.
 - d. Immerse the loop tip in the CaCl₂ and *vigorously* tap it against the wall of the tube to dislodge the cell mass. Hold the tube to the light to verify that the cell mass has been dislodged in the CaCl₂ solution.
5. Reflame the loop, cool it briefly in the ice, and place it on the lab bench.
6. Resuspend the cells in the "+" plasmid tube by repeatedly pipetting in

CAUTION! When using an open flame, use appropriate precautions. Make sure any loose clothing is secured, and tie long hair back. Do not lean over the flame.

(12) If the tubes are not immediately moved from the ice to the water bath, heat shock will not occur.

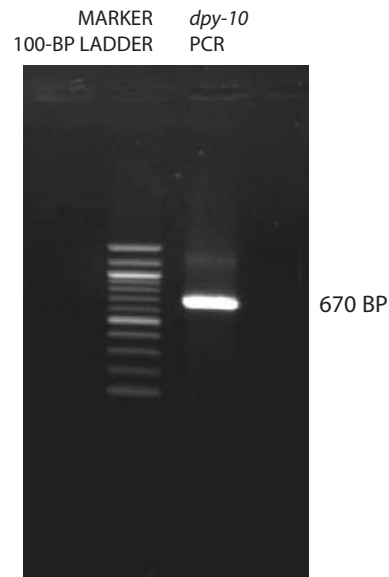
- and out, using a 100-1000 μL micropipettor with a sterile tip set at 200 μL .
- a. Pipette carefully to avoid making bubbles in suspension or splashing the suspension up the sides of the tube.
 - b. Hold the tube up to the light to check that the suspension is homogeneous. No visible clumps of cells should remain.
 - c. Place the tube on ice until you are ready to proceed to step 8.
7. Transfer a second mass of cells to the “-” plasmid tube as described in Steps 4 and 5 above. Resuspend the cells as described in step 6.
 8. Use a 1-10 μL micropipettor to add 5 μL of miniprep DNA solution (from Part IX) *directly into the cell suspension* in the “+” plasmid tube. Mix the contents of the tube by finger vortexing. Avoid making bubbles in the suspension or splashing the suspension up the sides of tube.
 9. Return the “+” plasmid tube to ice. Incubate both tubes on ice for an additional 15 minutes.
 10. While the cells are incubating on ice, use a permanent marker to label two LB plates and two LB/amp plates with your name (or group number) and the date.
 - a. Label one LB/amp plate “+.” This is the experimental plate.
 - b. Label the other LB/amp plate “-.” This is a negative control.
 - c. Label one LB plate “+.” This is a positive control.
 - d. Label one LB plate “-.” This is a positive control.
 11. Following the 15 minute incubation, heat shock the cells in the “+” plasmid and “-” plasmid tubes. *Note: it is critical that cells receive a sharp and distinct shock.*
 - a. Carry the ice beaker to the water bath. Remove the tubes from ice, and *immediately* immerse them in a 42°C water bath for 90 seconds.
 - b. Immediately return the tubes to the ice for at least 1 additional minute.
 - c. Leave the tubes at room temperature until you are ready to proceed to step 12.
 12. Place the tubes in a test tube rack at room temperature.
 13. Use a 100-1000 μL micropipettor and sterile tip to add 250 μL of LB broth to each tube. Mix the contents of the tube by gently tapping the tubes with your finger.
 - 14 Use a micropipettor with a sterile tip to add 100 μL of cell suspension from the “-” plasmid tube onto the “- LB” plate and another 100 μL onto the “- LB/amp” plate. *Do not allow the suspensions to sit on the plates too long before proceeding to Step 15 or the cell solution will soak*

into the agar before you have a chance to spread the cells.

15. Spread the cell solution over the surface of the plates either using a cell spreader or sterile glass spreading beads. Be sure to use sterile technique to avoid cross-contamination.
16. Use a micropipettor with a sterile tip to add 100 μ L of cell suspension from the "+" plasmid tube onto the "+ LB" plate and another 100 μ L onto the "+ LB/amp" plate. *Do not allow the suspensions to sit on the plates too long before proceeding to Step 17 or the cell solution will soak into the agar before you have a chance to spread the cells.*
17. Spread the cells as described in step 15. If used, remember to sterilize the cell spreader when done.
18. Allow the plates to sit at room temperature for several minutes to allow time for the cell suspension to absorb into the agar.
19. If spreading beads were used, invert the plates and gently tap bottom of the plate so the beads fall into plate lids. Carefully pour the beads from each lid into a waste beaker. Alternatively, the beads can be poured into a storage container to be washed and autoclaved for reuse.
20. Stack the plates (so they are all facing down) and tape them together to keep each group's experiment together.
21. Place the plates upside down in a 37°C incubator for 15-24 hours.
22. Check for transformants the next day. All transformants should now be HT115(DE3) containing the *dpy-10* feeding vector.
23. Follow the instructions found in the "RNAi by feeding" section to reduce the function of the gene with your newly created RNAi feeding strain.

RESULTS AND DISCUSSION

1. Describe the purpose of each of the following steps or reagents used during DNA isolation (Part I):
 - a. Freezing the worms
 - b. Adding Proteinase K.
 - c. Boiling.
2. Examine the results of your PCR reaction. Compare your results to the following sample gel.

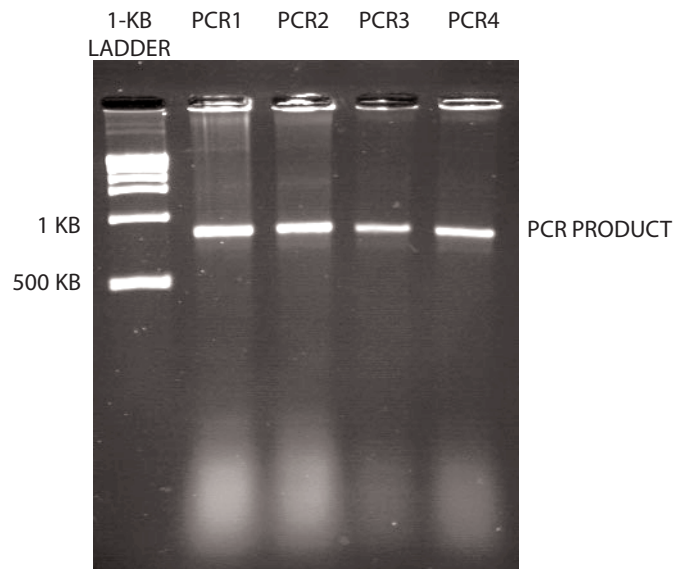


3. Did your PCR reaction work? Note: it is common to see diffuse (fuzzy) bands that run near the bottom of the gel. These bands are either leftover primer or "primer dimer," an artifact of the PCR reaction that results from the primers overlapping one another and amplifying themselves. The presence of primer dimer, in the absence of other bands, confirms that the reaction contained all components necessary for amplification.
4. Additional faint bands at other positions occur when the primers bind to chromosomal loci other than the *dpy-10* gene and give rise to "non-specific" amplification products. Do you think that nonspecific products could cause any problems? Why?
5. Taq polymerase adds an extra adenosine nucleotide to the 3' end of DNA that it amplifies. This extra "A" is added in a template-independent manner even when there are equal amounts of each nucleotide in the reaction mixture. How is this property of Taq polymerase utilized in the ligation reaction? How was the vector prepared to make this work? Reference: Marchuk *et al* (1991), *Nucleic Acids Res.* 19, 1154.
6. When cloning fragments into vectors, it is often important to make sure that the fragments are in the right orientation once ligated into

the vector. Will you be able to control the directionality of the cloning? That is, if compared to a reference gene such as the ampicillin gene, can you control whether or not the newly ligated gene reads the same direction as the ampicillin gene. For this experiment, do you think it will matter which direction the fragment is inserted into the vector? Why or why not?

7. Ligation buffer includes adenosine nucleotide (dATP). Why?
8. Briefly describe how bacterial cells are made competent. Why do they need to be heat shocked during the transformation? Put on ice? What is the purpose incubating in LB broth before plating the cells?
9. Re-examine the both sets of transformation plates. How well did the transformations work? Was the transformation with control DNA more or less efficient than the one with ligated DNA? Explain this similarity or difference.
10. Why are colonies picked from the LB plus ampicillin plate? What event does each colony represent?
11. Examine the gel from Part VII which possesses DNA that you amplified from transformed bacterial colonies. Based on the size of the bands of DNA in your gel, do any of the transformants have the expected insert? Explain how you determined this.

Here is an example gel:



12. Why does the newly created RNAi feeding vector have to be transformed into HT115(DE3) cells before it can be used to inactivate *dpy-10* by RNAi?

INFORMATION FOR INSTRUCTOR

CONCEPTS AND METHODS

This laboratory can help students understand several important concepts of modern biology:

- RNA interference as a method to inactivate genes by reverse genetics.
- Methods for isolating and purifying DNA.
- The movement from DNA sequence data by *in silico* computation to *in vitro* experimentation.
- Transcription and transcriptional control.

The laboratory uses several methods for modern biological research:

- DNA extraction and purification.
- Polymerase chain reaction (PCR).
- Ligation.
- Gel electrophoresis.
- Bioinformatics.

LAB SAFETY

Liquid nitrogen and dry ice, if used, are dangerous materials. The possibility of freeze burns represents a serious danger and is therefore of concern. **Appropriate precautions and safety equipment should be used.** Dangers include:

Nitrogen can spatter (possibly in eyes) while being poured.

Flying chunks of frozen objects could cause eye injury.

Students (being children) will want to reach out and touch nitrogen or other cold objects. As mentioned above, contact with nitrogen can cause tissue damage, and this *must* be prevented.

Therefore specific safety precautions should include:

Teachers must stress to their students the importance of not touching frozen objects, dry ice and liquid nitrogen. Wear goggles whenever pouring or dumping nitrogen or handling dry ice. Nitrogen or dry ice can spatter into the eyes, and potentially blinding pieces of frozen things can fly around when dropped.

Use a thermanl glove and/or tongs to handle any object going into or out of nitrogen or dry ice and to carry a nitrogen dewar.

In preparation for a worse-case scenario, teachers should familiarize themselves with the following first aid procedures for cryogenic freeze burns:

*If cryogenic liquid or cold objects contact a person's skin or eyes, frozen tissues should be flooded or soaked with tepid water (105-115°F, 41-46°C). **DO NOT USE HOT WATER.** Cryogenic burns that result in blistering or deeper tissue freezing should be promptly examined by a physician.*

Ethidium bromide is a known mutagen and potential carcinogen. Wear gloves when handling the stain and stained gels. Designate an ethidium bromide stain work area. Dispose of stain properly (see *DNA Science* by Micklos and Freyer, pp.256-257). In high school classrooms the teacher should handle the ethidium bromide stain.

Review all MSDS (Material Safety Data Sheets) for all chemicals and reagents in the lab before preparing and running the lab.

Never look at an unshielded UV light source. Wear a full face UV shield and cover all exposed skin if your UV light source is unshielded.

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
I. Isolate DNA	-7	15 min.	Pre-lab: Prepare overnight cultures of OP50 strain of <i>E. coli</i> grown in LB broth.
	-6	30 min.	Pre-lab: Seed NGM plates with OP50 <i>E. coli</i> strain (two per student group).
	-4	60 min.	Pre-lab: Chunk N2 worms to the appropriate plates in order to have worms growing prior to chunking on day 2.
	-2	10 min.	Pre-lab: Chunk N2 worms to OP50-seeded NGM plates in order to have worms for lab.
	1	60 min.	Pre-lab: Prepare and aliquot lysis buffer to tubes. Obtain liquid nitrogen or dry ice if needed. Make centrifuge adapters. Setup student stations
		20 min.	Lab: Setup worm lysis reactions
		120 min.	Lab: Freeze worms
II. Amplify DNA by PCR	1	15 min.	Pre-lab: Aliquot <i>dpy-10</i> primer/loading dye mix
		15 min.	Lab: Setup PCR reactions
		120 min.	Post-lab: Amplify DNA in thermal cycler
III. Analyze PCR Products by Gel Electrophoresis	2	15 min.	Pre-lab: Dilute TBE electrophoresis buffer
		30 min.	Pre-lab: Prepare agarose gel solution and cast gels
		15 min.	Lab: Load DNA samples into gels
	2 or 3	20+ min.	Lab: Electrophore samples
		20+ min.	Post-lab: Stain gels
		30-45 min.	Post-lab: De-stain gels (for CarolinaBLU™)
	20 min.	Post-lab: Photograph gels	
	30 min.	Post-lab: Determine if PCR was successful	
IV. Ligate PCR Product	3	20 min.	Pre-lab: Prepare and aliquot ligase mix
		20 min.	Lab: Prepare ligation reactions
		120 min.	Post-lab: Incubate ligation reaction at room temperature overnight
V. Prepare Competent Cells	1	10 min.	Pre-lab: Streak a plate with DH5α <i>E. coli</i> cells on an LB plate. Optional: plate to an LB plus ampicillin plate to ensure that the cells are sensitive to LB.
	2	10 min.	Pre-lab: Begin an <i>E. coli</i> culture in 5 mL of LB broth from the streaked plate of DH5α and grow overnight in a 37°C shaker.
	3	10 min.	Pre-lab: Approximately 2-4 hours before the lab, inoculate an <i>E. coli</i> culture with 1 mL of DH5α overnight culture per 100 mL of LB broth.
VI. Transform Ligated DNA into Competent Cells	3	60 min.	Lab: Transform and plate cells.
VII. Perform Single Colony PCR and Inoculate Overnight Cultures	4	15 min.	Pre-lab: Aliquot <i>T7</i> primer/loading dye mix prepare LB plus ampicillin. Aliquot LB plus ampicillin to snap cap tubes (4 per student group).
		60 min.	Lab: Setup PCR reactions and overnight cultures. Inoculate overnight cultures.
		120 min.	Post-lab: Amplify DNA in thermal cycler

Part	Day	Time	Activity
VIII. Analyze PCR Products by Gel Electrophoresis	5	15 min.	Pre-lab: Dilute TBE electrophoresis buffer
		30 min.	Lab: Prepare agarose gel solution and cast gels
		15 min.	Load DNA samples into gels
		20+ min.	Electrophorese samples
		20+ min.	Post-lab: Stain gels
		30-45 min.	De-stain gels (for <i>CarolinaBLU</i> [™])
IX. Perform Plasmid Miniprep	6	20 min.	Photograph gels
		30 min.	Determine if PCR was successful
IX. Perform Plasmid Miniprep	6	40 min.	Pre-lab: Prepare fresh SDS/NaOH solution. Prepare aliquots of GTE, KOAc, isopropanol, 95% EtOH and TE
		60 min.	Lab: Purify plasmid DNA.
X. Transform Vector into RNAi Feeding <i>E. coli</i> Strain	6	60 min.	Pre-lab: Streak HT115(DE3) <i>E. coli</i> to LB plates (one per team) and place in a 37°C incubator overnight. Prepare LB and LB plus ampicillin plates (two per team)
			7
	8	60 min.	Lab: Perform transformation
		10 min.	Lab: Examine results
			Innoculate LB plus ampicillin broth with HT115(DE3) transformed with the RNAi feeding vector and proceed to RNAi by feeding

I. WORM DNA PREPARATION

Pre-lab Preparation

Prior to carrying out the experiments in this protocol, it is necessary to grow the *C. elegans* strain from which DNA will be isolated. This requires some planning and preparation. One approach would be to have students grow the worms prior to beginning these experiments. You (or the students) will need to prepare enough plates with wild-type worms grown on OP50 to have worms for each group of students.

Prepare 1X PCR buffer by adding 90 µL of distilled or deionized water to 10 µL of 10X PCR buffer (100 mM Tris, 500 mM KCl, 15 mM MgCl₂ pH 8.3). Prepare worm lysis buffer by adding 95 µL of 1X PCR buffer to 5 µL of 20 mg/mL proteinase K.

For each group of students, aliquot 10 µL of lysis buffer into either a 0.2 mL or 0.5 mL tube (whichever size is accommodated by your thermal cycler). *The proteinase K should be added the day of the experiment and the lysis buffer should be stored on ice once proteinase K has been added. Alternatively, the proteinase K can be added following the –80°C freezing step if the samples will be stored for a prolonged period of time.*

Obtain enough dry ice, liquid nitrogen or access to a –80°C freezer to freeze all the samples. *Dry ice and liquid nitrogen are very cold and can cause tissue damage. Avoid contact with these reagents and with any cold vapors emanating from them. Use dry ice sparingly in a ventilated area to avoid toxic levels of carbon dioxide.*

Remove the caps from 1.5 mL tubes to use as adaptors in which to centrifuge 0.5 mL PCR tubes. Two adaptors are needed to spin 0.2 mL PCR tubes (a capless 0.5 mL PCR tube nested *within* a capless 1.5 mL tube).

Pre-lab Set Up for DNA Isolation (per student or student group)

Lysis solution 10 μ L (in a 0.2 or 0.5 mL tube, depending on the thermal cycler)

Permanent marker
Micropipets and tips (1-10 μ L)
Microcentrifuge tube rack
Worm pick
Dissection microscope
Bunsen burner
A plate with adult wild-type worms grown on worm plates seeded with OP50
PCR tubes (0.2 mL or 0.5 mL)

Shared Items

Microcentrifuge
Microcentrifuge adapters for 0.2 mL or 0.5 mL PCR tubes
Container of liquid nitrogen or dry ice (or access to a -80°C freezer)
Thermal cycler or hot water bath (65°C and 90°C)

II. AMPLIFY DNA BY PCR

The primer/loading dye mix incorporates the appropriate primer pair (0.26 picomoles/ μ L of each primer), 13.8% sucrose, and 0.008% cresol red. The inclusion of loading dye components (sucrose and cresol red) allows the amplified product to be directly loaded into an agarose gel for electrophoresis following PCR. Each Ready-To-Go™ PCR Bead contains reagents such that in a final volume of 25 μ L the reaction contains 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and 200 μ M of each dNTP.

The lyophilized *Taq* DNA polymerase in the bead becomes active immediately upon addition of the primer/loading dye mix and template DNA. In the absence of thermal cycling, “nonspecific priming” at room temperature allows the polymerase to begin generating erroneous products, which can show up as extra bands during gel analysis. *Therefore, work quickly. Be sure the thermal cycler is ready and have all students setup their PCR reactions as a coordinated effort. Add the primer/loading dye mix to all PCR bead tubes. Then, add each student DNA template, and begin thermal cycling as quickly as possible. Hold PCR reactions on ice until all are ready to load into the thermal cycler.*

PCR amplification from crude cell extracts is biochemically demanding and requires the precision of automated thermal cycling. However, amplification of the *dpy-13* locus is not complicated by the presence of repeated DNA units. Therefore, the recommended amplification times and temperatures will work adequately for all types of thermal cyclers.

Pre-lab Preparation

Aliquot 25 μ L of the *dpy-10* primer/loading dye mix per student. The primer/loading dye mix may collect in the tube cap during shipping; pool the reagent at the bottom of the tube by spinning the tube briefly in a microcentrifuge or by sharply tapping the bottom of the tube on the lab bench.

Pre-lab Set Up for DNA Amplification (per student or student group)

Worm DNA. 2.5 μ L (from Part I)
dpy-13 primer/loading dye mix, 30 μ L
Ready-To-Go™ PCR beads (in a 0.2 mL or 0.5 mL PCR tube)
Permanent marker
Micropipet and tips (1-10 and 10-100 μ L)
Microcentrifuge tube rack
Container with cracked or crushed ice

Shared Items

Mineral oil, 5 mL (depending on thermal cycler)
Thermal cycler

III. ANALYZE AMPLIFIED DNA BY GEL ELECTROPHORESIS

The cresol red and sucrose in the primer mix function as loading dye, allowing amplified DNA samples to be loaded directly into an agarose gel. Although this is a nice time saver, the cresol red loading dye is more difficult to use than typical loading dye because it has relatively little sugar and cresol red is a fainter dye. So, encourage students to load carefully.

1-kb ladder and 100-bp ladder are used as DNA size markers in this experiment. The smallest band in the 100-bp ladder is 100 bp and each higher band is 100 bp larger (i.e. 200, 300, 400 bp, etc.). The size of the DNA fragments in the 1-kb ladder (from smallest to largest) are 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 kb.

View and photograph gels as soon as possible after appropriate staining/destaining. Over time at room temperature, the smaller PCR products will diffuse through the gel and the corresponding bands will lose sharpness.

Pre-lab Preparation

Prepare a 1X concentration of TBE by adding 25 mL of 20X concentrated stock to 475 mL of deionized or distilled water for each student group. Mix thoroughly.

Prepare a 1% agarose solution by adding 1 g of agarose per 100 mL of 1X TBE in a 500 mL flask or beaker. Prepare enough 1 X TBE agarose solution to have one gel per student group. Heat the flask or beaker in a boiling water bath (approximately 15 minutes) or in a microwave oven (approximately 4 minutes) until the agarose is *completely* dissolved. You should no longer see agarose particles floating in the solution when the beaker is swirled. Allow the agarose to cool to approximately 55°C in a hot water bath.

Pre-lab Set Up for Gel Analysis (per student)

Amplified *dpy-10* PCR product from Part III (store on ice)
Container with cracked or crushed ice

Shared Items

100-bp ladder, 5 µL per row of gel (thaw and store on ice)
1% agarose in 1X TBE (hold at 55°C), 50 mL per gel
1X TBE buffer, 300 mL per gel
Ethidium bromide (1 µg/mL), 250 mL
or
*CarolinaBLU*TM Gel & Buffer Stain, 7 mL
*CarolinaBLU*TM Final Stain, 250 mL

Micropipet and tips (1-10 µL)
Microcentrifuge tube rack
Gel electrophoresis chambers
Power supplies
Water bath for agarose solution (55°C)
Latex gloves
Staining tray
UV Transilluminator
Digital or instant camera (optional)
Casting tray, comb, and gel electrophoresis box
Masking tape (if necessary)

CarolinaBLU™ STAINING

POST-STAINING

1. Cover the electrophoresed gel with the *CarolinaBLU™* Final Stain and let sit for 20–30 minutes. Agitate gently (optional).
2. After staining, pour the stain back into the bottle for future use. (The stain can be used 6–8 times.)
3. Cover the gel with deionized or distilled water to destain. Chloride ions in tap water can partially remove the stain from the DNA bands and will cause the staining to fade.
4. Change the water 3 or 4 times over the course of 30–40 minutes. Agitate the gel occasionally.
5. Bands that are not immediately present will become more apparent with time and will reach their maximum visibility if the gel is left to destain overnight in just enough water to cover the gel. Gels left overnight in a large volume of water may destain too much.

PRE-STAINING

CarolinaBLU™ can also be used to stain the DNA while it is being electrophoresed. Pre-staining will allow students to visualize their results prior to the end of the gel run. However, post-staining is still required for optimum viewing.

To pre-stain the gel during electrophoresis, add *CarolinaBLU™* Gel and Buffer Stain in the amounts indicated in the table below. Note that the amount of stain added is dependent upon the voltage used for electrophoresis. *Do not use more stain than recommended. This may precipitate the DNA in the wells and create artifact bands.*

Gels containing *CarolinaBLU™* may be prepared one day ahead of the lab day, if necessary. However, gels stored longer tend to fade and lose their ability to stain DNA bands during electrophoresis.

Use the table below to add the appropriate volume of *CarolinaBLU™* stain to the agarose gel:

Voltage	Agarose Volume	Stain Volume
<50 Volts	30 mL	40 µL (1 drop)
	200 mL	240 µL (6 drops)
	400 mL	520 µL (13 drops)
>50 Volts	50 mL	80 µL (2 drops)
	300 mL	480 µL (12 drops)
	400 mL	640 µL (16 drops)

Use the table below to add the appropriate volume of *CarolinaBLU™* stain to 1× TBE buffer:

Voltage	Agarose Volume	Stain Volume
<50 Volts	500 mL	480 µL (12 drops)
	3000 mL	3 mL (72 drops)
>50 Volts	500 mL	960 µL (24 drops)
	2600 mL	5 mL (125 drops)

IV. LIGATE PCR PRODUCT TO VECTOR

Pre-lab Preparation

Obtain fresh 10X ligation buffer/ATP solution. ATP is somewhat unstable in solution, so do not use old buffer/ATP and take care to keep the solution frozen when not in use. T-tagged vector is also critical to the experiment and should be stored frozen until needed.

T4 DNA ligase is critical to the experiment and rather expensive. Make one aliquot of the ligase mix sufficient for all experiments by combining 5 μ L distilled water, 2 μ L T-tagged, *EcoRV*-cut L4440 vector, 2 μ L 10X ligation buffer and 1 μ L ligase per student group, plus enough for two others (one for the instructor and a little extra in case of errors). Hold the ligation mix on ice during the laboratory. The ligase mix may be dispensed directly into each experimenter's reaction tube by the instructor if there is concern the students will waste or mishandle it.

Pre-lab Set Up for Ligation (per student)

Amplified PCR products from Part I (store on ice)
Container with cracked or crushed ice
Ligase mix (10 μ L)
Micropipet and tips (1-10 μ L)
1.5 mL microcentrifuge tubes
permanent marker
microcentrifuge tube rack
0.5 mL PCR tubes (optional)

Shared equipment

Microcentrifuge (optional)

V. PREPARE COMPETENT CELLS

Pre-lab Preparation

Sterilize 50mM CaCl_2 and LB broth by autoclaving or filtering through a 0.45 or 0.22 μ m filter.

Two days before the lab, prepare a streaked LB plate of DH5 α *E. coli* cells. The day before the experiment, use this fresh streaked plate to inoculate an overnight culture of LB broth with a colony of DH5 α .

Approximately 2-4 hours before the lab, inoculate an *E. coli* culture with 1 mL of overnight culture per 100 mL of LB broth. Make sure the culture is well aerated by growing the culture in a flask at least five times the volume of the culture. Grow at 37°C with shaking for 2 hours and 15 minutes, at which point the cells should be in mid-log phase. Hold the cells in mid-log phase by storing the culture on ice for up to 2 hours. Each group will need 20 mL of mid-log cell culture.

Pre-lab Set Up for preparation of competent cells (per student)

CaCl_2 (50mM) (15mL)
Mid-log DH5 α cells (two 10 mL cultures)
Micropipet and tips (100-1000 μ L)
Container with cracked or crushed ice
Permanent marker
Waste Beaker
Test tube rack

Shared equipment

Clinical centrifuge (2000-4000rpm)
Shaking incubator (37°C)
Spectrophotometer (optional)
Sterile pipettes (5 or 10 ml) and pipette aid or bulb
Paper towels

VI. TRANSFORM LIGATED DNA INTO COMPETENT CELLS

It is important that the steps in the transformation be followed very carefully to ensure success. Emphasize the importance of keeping the cells on ice and heat shocking the cells abruptly, as these steps are critical to the protocol.

Pre-lab Preparation**Pre-lab Set Up for Transformation** (per student)

Ligated DNA from Part IV (store on ice)
L4440 *dpy-10* DNA (control, 4 µL) (store on ice)
Container with cracked or crushed ice
Competent cells from part V (2 tubes with 100 µL each, thaw and store on ice)
Micropipet and tips (1-10 and 100-1000 µL)
1.5 mL microcentrifuge tubes
permanent marker
microcentrifuge tube rack
2 LB and 2 LB+Amp plates
LB broth (2 mL)

Shared equipment

Microcentrifuge (optional)
Water bath (37°C and 42°C)
Masking tape
Shaking incubator (37°C) (optional)
Beaker of 95% ethanol, cell spreader, and Bunsen burner
or
Sterile glass spreading beads

VII. PERFORM SINGLE COLONY PCR AND INOCULATE OVERNIGHT CULTURES

In order to make sure that the transformants contain the correct insert in the cloning site, it is necessary to amplify the DNA in the vector from transformants. In order to have DNA from the transformants, it is most convenient to inoculate overnight cultures at the same time that the colonies are picked for PCR.

Pre-lab Preparation

Prepare LB plus ampicillin by adding 10 mL of sterile-filtered 10 mg/mL ampicillin per liter of cooled sterile LB.

Pre-lab Set Up for single colony PCR and inoculation of overnight cultures (per student)

LB+amp plate with transformants marked "L" (Part IV)
Aliquot T7 primer/loading dye mix (30 µL)
1.5 mL microcentrifuge tube
Micropipet and tips (10-100 µL)
permanent marker

microcentrifuge tube rack
15 mL snap cap tubes (4) containing 5 mL LB plus ampicillin each
Microcentrifuge tube rack
Test tube rack
Container with cracked or crushed ice
Ready-To-Go™ PCR beads (in a 0.2 mL or 0.5 mL PCR tube) (4 per group)

Shared equipment

Thermal cycler
Shaking incubator (37°C)
Mineral oil, 5 mL (if necessary for the thermal cycler)

VIII. ANALYZE AMPLIFIED DNA BY GEL ELECTROPHORESIS

Follow the instructions for Part III, above.

Pre-lab Set Up for Gel Analysis (per student)

Amplified DNA from part VII (store on ice)
Container with cracked or crushed ice

Shared Items

1-kb ladder, 5 µl per row of gel (thaw and store on ice)
1% agarose in 1X TBE (hold at 55°C), 50 mL per gel
1X TBE buffer, 300 mL per gel
Ethidium bromide (1 µg/mL), 250 mL
or
CarolinaBLU™ Gel & Buffer Stain, 7 mL
CarolinaBLU™ Final Stain, 250 mL

Micropipet and tips (1-10 µL)
Microcentrifuge tube rack
Gel electrophoresis chambers
Power supplies
Water bath for agarose solution (55°C)
Latex gloves
Staining tray
UV Transilluminator
Digital or Polaroid camera (optional)
Casting tray, comb, and gel electrophoresis box
Masking tape (if necessary)

IX. PERFORM PLASMID MINIPREP

In order to get the feeding vector into HT115(DE3) it is necessary to purify plasmid DNA from the DH5α transformants and then transform this DNA into HT115(DE3). For a complete description of the plasmid DNA purification, see DNA Science by Micklos and Freyer, pp.423-430.

Pre-lab Preparation

Make fresh 1% SDS/0.2N NaOH: Mix 1 mL of 10% Sodium Dodecyl Sulfate(SDS) and 0.5 ml of 4N NaOH into 8.5 ml of distilled water per 10 mL needed. Always use fresh SDS/NaOH solution. If a precipitate forms, warm solution in a water bath, and shake gently to dissolve precipitate.

Pre-lab Set Up for Plasmid DNA purification (per student)

E. coli cultures of transformants from Part VII
Ethanol (100%) (1mL)
Glucose/Tris/EDTA (GTE) (500 μ L)
Isopropanol (500 μ L)
Potassium acetate/acetic acid (KOAc) (800 μ L)
SDS/sodium hydroxide (SDS/NaOH) (1 mL)
Tris/EDTA (TE), 75 μ L
Micropipet and tips (10-100 and 100-1000 μ L)
Microcentrifuge tube rack
Container with cracked or crushed ice
1.5 mL microcentrifuge tubes (2)
Permanent marker

Shared equipment

Microcentrifuge

X. TRANSFORM VECTOR INTO RNAi FEEDING *E. coli* STRAIN**Pre-lab Preparation**

The day before the laboratory, streak HT115(DE3) bacteria on LB agar plates for each group. These “starter plates” will be ready to use following overnight incubation at 37°C.

Be sure to have a streaked plate or stab/slant culture of viable *E. coli* cells from which to streak starter plates. Also, streak the *E. coli* strain on an LB/amp plate to ensure that an ampicillin-resistant strain has not been used by mistake.

Sterilize 50 mM calcium chloride (CaCl_2) solution and LB broth by autoclaving or filtering through a 0.45- μ m or 0.22- μ m filter (Nalgene or Corning). To eliminate autoclaving completely, store filtered solutions in presterilized 50-mL conical tubes.

Pre-lab Set Up for Gel Analysis (per group)

Miniprep DNA (from part IX)*
 CaCl_2 (50mM) (600 μ L)
HT115(DE3) *E. coli* grown overnight on LB plates
LB Broth (600 μ L)
2 LB and 2 LB+AMP plates

Supplies and Equipment

Micropipet and tips (1-100 and 100-1000 μ L)
Microcentrifuge tube rack
Container with cracked or crushed ice
Beaker of 95% ethanol and cell spreader (or spreading beads)
Inoculating loop
Bunsen burner
Test tube rack
Permanent marker
Culture tubes (two 15 mL)
Water bath (37°C and 42°C)
37°C incubator
Masking tape

ANSWERS TO DISCUSSION QUESTIONS

1. Describe the purpose of each of the following steps or reagents used in DNA isolation (Part I):
 - a. **Freezing the worms helps to disrupt the cuticle of the worms, making it easier to extract the DNA.**
 - b. **Proteinase K is an enzyme that digests protein, including the cuticle, which also helps to extract the DNA.**
 - c. **Boiling denatures proteins, including the Proteinase K, which could damage the Taq polymerase in the PCR reaction, and DNases, that would damage the DNA template needed for PCR.**
4. Do you think that nonspecific products could cause any problems? **Non-specific bands could be cloned into the vector, as there is no PCR product purification step. This will make it harder to get the desired clone. Hence, it is undesirable to have any non-specific product. One way to minimize these bands would be to raise the annealing temperature during the PCR reaction.**
5. Taq polymerase adds an extra "A" to the 3' of DNA that it amplifies. This "A" is added in a template-independent way when there are equal amounts of dNTPs in the reaction mixture. How is this property of Taq polymerase used in the ligation reactions? **The extra "A" at the 3' end of the amplified fragment will create a 3' overhang. How was the vector prepared to make this work? Using Taq polymerase, an extra "T" was added to blunt-end cut vector DNA. This is done by forcing Taq to add only Ts by including only dTTP in the reaction mix. The 3' T's are complementary to the 3' A's that are added to the PCR product, greatly increasing the efficiency of ligation into the vector. In addition, the 3' Ts make it impossible for the vector to religate to itself, thereby forcing ligation to the PCR product.**
6. When cloning fragments into vectors, it is often important to make sure that the fragments are in the right orientation once ligated into the vector. Will you be able to control the directionality of the cloning? **No. The vector has 3' T's at both ends and the insert has 3' A's at both ends, so ligase will ligate the two molecules together in either orientation. Do you think it will matter which direction the fragment enters the vector for this experiment? No. Why or why not? The purpose of the vector is to transcribe RNA from both strands using T7 promoters upstream of the insertion site on both strands. This will be accomplished when the PCR product is inserted into the vector in either orientation.**
7. Ligation buffer includes ATP. Why? **ATP is a cofactor for the ligation reaction. It is converted into PPi and AMP during the reaction.**
8. How are bacterial cells made competent? **Bacterial cells are made competent for transformation by freezing them in a solution that contains cations. It is thought that the cations coat the surface of the cell membrane and the DNA, neutralizing the negative charges that normally would lead to repulsion. Why do they need to be heat shocked during transformation? Empirically, heat shock enhances transformation. The heat shock probably leads to a thermal gradient that helps pull the DNA into the cells. Put on ice? The ice prior to heat shock is thought to reduce motion, allowing the DNA and cells to come into close contact. The ice after the transformation is thought to help the cells recover from the heat shock. What is the purpose of adding LB and incubating before plating the cells? LB is bacterial food. It allows the cells time to transcribe and translate the AmpR gene and transcript so that the cells will be resistant to the ampicillin on the selective plates.**
9. Examine the plates from the transformations. Did the transformations work? **If there are colonies growing on the LB plus ampicillin plates and nothing growing on the LB plates, the transformation worked. Was the transformation with control DNA more or less effective than the one with ligated DNA? Explain this similarity or difference. The transformation with control DNA should give many more transformants than the ligation. Ligations generally give rise to a small number of circular**

molecules that can transform the bacteria. The control DNA is intact plasmid and there are many copies, all capable of transforming the bacteria. This translates into more colonies on the control LB plus ampicillin plate.

10. Why are colonies picked from the LB plus ampicillin plate? **The ampicillin selects against non-transformed cells, only allowing transformed cells to reproduce.** What event does each colony represent? **Each colony is made up of descendants from a single bacterium that was transformed with the vector.**
11. Based upon the size of the control DNA, do any of the transformants have the expected insert? Explain how you determined this. **The PCR fragment is 670bp long. The 5' end of the T7 primer will anneal 37bp from the site where EcoRV cuts the vector at one end and 74 bp from the site where EcoRV cuts the other side. In total, the size is therefore 670+37+64, or 771bp. As these fragments are close to the size of the PCR insert, students can compare their results with the original primer pair to their results from the T7 PCR to see if they have the correct insert.**
12. Why does the newly created RNAi feeding vector have to be transformed into HT115(DE3) cells before it can be used to inactivate *dpy-10* by RNAi? **HT115(DE3) contains a copy of the gene that codes for T7 RNA polymerase. T7 RNA polymerase is what will transcribe the RNA from both strands of the insert, starting at the T7 RNA polymerase promoter sequences.**