Appendix 2. Student Lab Manual

Week One: Using Zebrafish as a Developmental Model System

Zebrafish, or *Danio rerio*, are an excellent model system for development and genetics. Some of the advantages over other systems include a large number (> 150) of embryos that are externally fertilized, optical transparency, and rapid development. The objectives of this week's laboratory are to identify some of the resources available for studying zebrafish and to observe developmental stages of zebrafish embryos.

Part One: Zebrafish Resources

1. The zebrafish community has a website full of invaluable information. The address is www.zfin.org. Go there, click under "Anatomy Atlases and Resources". Click on "Movies of Development". Click and watch the movie.

a. Name at least one anatomical structure you can identify at the end of the movie.

b. This movie is sped up. What is the amount of time lapsed in the entire movie?

2. Go back to "Anatomy Atlases and Resources". Click on "Developmental Staging Series" to answer the following three questions. As you click on a stage, you can read information and click on the figures to see pictures of each stage.

a. What is the optimal temperature for zebrafish growth (and therefore, this staging series)?

b. The zebrafish heart is beating by which stage?

3. On the homepage of zfin, click on "Genes/Markers/Clones". Type in lin28a into the "Name/Symbol" search box. Click on "1 Gene". *Which chromosome is lin28a on in zebrafish?*

4. Under "Gene Expression", to the right of "Directly Submitted Expression Data", click on "6 figures". These images are collected using a technique called RNA *in situ* hybridization. Without going into the details, this technique stains the mRNA for the *lin28a* gene dark purple.

a. At bud stage, where is the lin28a *mRNA?*

b. At 24 hours post fertilization (hpf), lin28a *mRNA is expressed the most in which organ system? Hint: you may have to go back to the "Developmental Staging Series" to correlate the time with the stage.*

c. If we stained the embryo for lin28a *DNA instead, would the results differ? Why or why not?*

5. Go back to the lin28a page. Under "phenotype", to the right of "Data", click on "4 figures". Click on "Figure 3" (Ouchi et al., 2014). In this figure, the authors used a chemical called a morpholino (MO) to reduce the amount of Lin28a protein. Fish embryos treated with the morpholino are called morphants. The authors then restored the amount of Lin28a protein in a morphant by co-injecting mRNA encoding *lin28a*; this is called a rescue experiment. If the change in phenotype of the morphant is due to changes in Lin28a protein amounts and not an off-target effect, then the phenotype of the rescued embyros should be comparable to that of the negative control.

a. Using panel G, write one or two sentences to describe what happened to these morphant fish as compared to the control in your own words.

b. In panel H, the authors quantified their data. Which has the largest percentage of dead embryos: 1) treatment with lin-28a *MO, 2) control MO, or 3)* lin-28a *MO + mRNA?*

c. Name and identify at least one negative control in the experiment depicted in panel H. What is the purpose of this negative control? Were all the embryos normal in the negative control?

d. In panel H, the data are represented as percentages instead of raw numbers. Why was this a better choice?

6. Go back to the lin28a page. At the bottom of the page, click on the "CITATIONS" link. Click on the paper by Ramachandran et al., 2010. *Based on the first sentence of this abstract, in your own words, what makes fish different than us?*

Part Two: Live Zebrafish Analysis

7. Look at the two fish to the right. Males are generally skinnier and pinker than the female fish. Females often have a yellow dorsal fin. *Is the female at the top or at the bottom?*

8. You have a dissecting microscope and a tray of zebrafish embryos with several wells labeled. Identify the approximate age of the fish in each well (less than 24 hours, 1 day post fertilization (dpf), 2 dpf, 4 dpf).

A.
B.
C.
D.

9. Examine the youngest embryos. They were fertilized this morning.

a. Draw a picture of what you see in your lab notebook.

b. Label the chorion, the yolk, and the embryo.

c. Does this embryo have more or less than 1000 cells? (Hint: Use the "Developmental Staging Series".)

d. How old (hours post fertilization) do you think this embryo is?

10. With your partner, use the camera to capture an image of an embryo that is approximately 2 dpf. *Show your instructor the image and indicate the eye and the ear.*

11. *How does the size of the yolk change with the age of the fish? Why?*

12. Thinking about how organisms develop, would you expect the heart to beat faster in an older, more developed embryo or in a younger, less developed embryo? *Write your prediction in your notebook.*

From the front of the room, collect 2 different plates of embryos: one at 2 dpf and one that is a different age. Find the beating heart in the 2 dpf embryos, and zoom in.

a. While your partner times you, count the number of heartbeats in 10 seconds. *Record the number in your notebook.*

b. Find another fish and count the number of heartbeats. *Record the number in your notebook.*

c. Repeat step b until you have counted 3 different fish. *Record the number in your notebook.*

d. This is the number of heartbeats for 10 seconds. How would you determine the beats per minute? Enter these three data points of beats per minute into Excel.

e. Using the differently aged embryos, repeat the steps until you have three additional data points for these embryos. *Record the numbers in your notebook.*

g. **With your partner, generate a complete figure (graph with error bars and figure legend) comparing the beats per minute for the two differently aged fish embryos for submission.**

Appendix 2. Student Lab Manual

Week One: Journal Club Homework

For your next laboratory period, you should prepare for lab by reading sections and answering questions from the following two review articles.

Lush, M.E., and Piotrowski, T. (2014a). Sensory hair cell regeneration in the zebrafish lateral line. Dev. Dyn. *243*, 1187–1202.

Stoick-Cooper, C.L., Moon, R.T., and Weidinger, G. (2007). Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. Genes Dev. *21*, 1292–1315.

Make sure you download these papers before leaving lab. I strongly suggest that you input them directly into Mendeley and that you bring your answers with you to lab next week. You may collaborate and discuss your answers with others. Do not wait until the last minute to do this, as reading these papers is more challenging than reading your textbook.

For **Lush and Piotrowski, 2014**, read the following sections:

Introduction
Anatomy of the Lateral Line
Cellular Aspects of Zebrafish Hair Cell Regeneration
Conclusions
Figure 1

1. *What is a hair cell, and where are they found?*

2. *How does the regenerative capacity of mammalian hair cells differ from that of non-mammalian organisms? What happens to a mammal when hair cells are damaged in an adult?*

3. *What do the authors state as a goal of understanding regeneration in non-mammalian systems?*

4. *Define lateral line, neuromast, otic vesicle, and stereocilia.*

5. *Name three different compounds that are ototoxic to zebrafish.*

6. *After treatment to destroy hair cells in a zebrafish larva, how many <u>days</u> will it take for the hair cells to regenerate?*

7. *Examine figure 1. The blue-violet spots are neuromasts. How were they stained?*

8. *In figure 1A, how many neuromasts do you see in the tail?*

9. *In your own words, describe what happened in panels E, F, and G.*

For **Stoick-Cooper et al., 2007**, read the following sections:

What is regeneration?
Fin regeneration (including positional memory and summary)

10. *In your own words, give an example of a short-lived cell type in humans.*

11. *How do mammals differ from amphibians and teleost fish in what they can regenerate?*

12. *Define regeneration, blastema, progenitor cell, morphogenetic, urodele, dedifferentiate, quiescent, and positional memory.*

13. *What structures can zebrafish regenerate?*

14. *What makes zebrafish a better model than an axolotl?*

15. *What types of tissues are present in the caudal fin?*

16. *What are the three steps of fin regeneration? Using figure 2, describe which image depicts each of these three steps.*

17. *How fast does fin regeneration occur, and what affects this rate directly? Why is this a factor?*

Week Two: Genotyping Zebrafish Part One

Background

Lin28a is hypothesized to play a role in regeneration by enhancing cellular metabolism (Figure 1). More active mitochondria lead to an increase in ATP production, mimicking the high energy state present during embryonic development. We predict that Lin28a directly contributes to the energy necessary for cell growth and division present in regeneration. Therefore, a mutant zebrafish lacking the *lin28a* gene will have problems with regeneration.

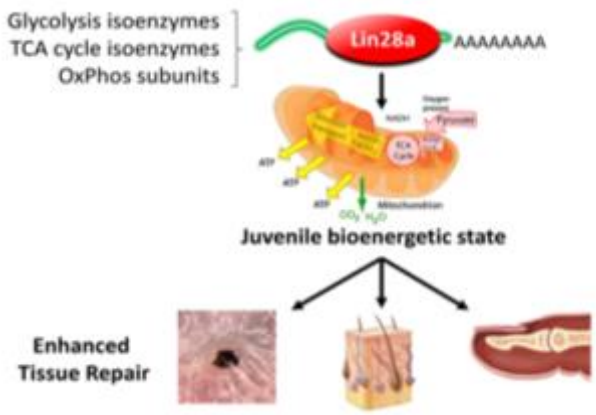


Figure 1. Lin28a can increase transcription of genes necessary for metabolism. Adapted from http://www.sciencedirect.com/science/article/pii/S009286741301278 6

In order to make a zebrafish with a nonfunctional *lin28a* gene, CRISPR/Cas9 technology was used (watch the video here for an explanation: https://www.youtube.com/watch?v=2pp17E4E-O8).

1. *Where did CRISPR/Cas9 systems originate, as in what kind of organisms use this system as a part of their natural (not genetically engineered) life? What is the purpose of CRISPR/Cas9 in these organisms?*

Basically, **CRISPR/Cas9** is an enzymatic tool that cuts genomic DNA at a specific sequence, in our case, at the *lin28a* gene. The cell then repairs the cut DNA incorrectly. This generates a mutation in the gene encoding the Lin28a protein. (Notice that in vertebrate systems, the gene name is lowercase and italicized and the protein name is capitalized but not italicized.) In the zebrafish we have available, the mutation has been **sequenced** (read letter by letter) and revealed to be an 11-base pair deletion (Figure 2).

```
WT   301  TTACAAGTGACGGGGCCTGGAGGGGCACCATGTGTGGGCAGCGAGAAGAAACCTAAGGGT  360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MUT  301  TTACAAGTGACGGGGCCTGGAGGGGCACCATGTGTGGGCAGCGAGAAGAAACC-------  353

WT   361  ACCCAAAAGAGGCGGTCAAAAGGAGATCGATGCTTCAACTGTGGAGGACCCAATCATCAT  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MUT  354  ----AAAAGAGGCGGTCAAAAGGAGATCGATGCTTCAACTGTGGAGGACCCAATCATCAT  409
```

Figure 2. A portion of the wild-type (WT) *lin28a* gene is contrasted to the CRISPR mutant (MUT) *lin28a* gene. Nucleotides 301 to 420 (WT numbering) are presented. Hyphens represent deleted nucleotides in the mutant sequence. Wherever a vertical line connects the two sequences, the nucleotide is identical. (Note well: although this is presented as multiple lines of sequence, it is one continuous line so the next letter after C at the end of the MUT line at the top is the A at the start of the MUT line at the bottom.)

Cut out figure 2 and tape or glue it into your lab notebook.

Conveniently for us, the region that was deleted contains a single restriction site specific for the restriction enzyme KpnI (GGTACC). Restriction endonucleases (or ***restriction enzymes***) are some of the most powerful tools in modern molecular biology. These enzymes, produced by bacteria, interact with ***restriction sites***, highly specific nucleotide sequences in DNA molecules (Figure 3). They then cut both strands of the double-stranded DNA, creating DNA restriction fragments. Because the restriction sequence is very specific and different DNAs will have different numbers of these sequences, digestion of different DNAs with the same enzyme may yield differently sized restriction fragments. This restriction analysis can be used as a fingerprint to distinguish DNA molecules from each other. This analysis is referred to as **restriction length fragment polymorphism (RFLP)** and is commonly used in genetic testing. *Highlight the KpnI site in the WT sequence taped in your notebook.*

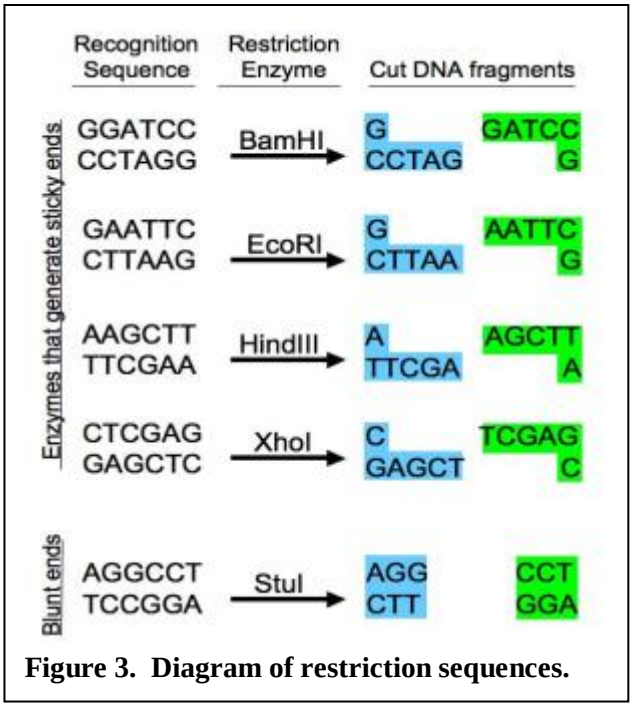


Figure 3. Diagram of restriction sequences.

That means that we can isolate genomic DNA from the zebrafish tail fin and use the KpnI restriction enzyme to see if the DNA is cut or not to determine if that individual fish contains two mutant genes (**homozygous**) or two WT genes (**homozygous**) or if it contains one gene of each (WT and mutant; **heterozygous**). We do not know which individuals have mutant genes and which do not so your job is to determine which individuals are which over the next three weeks. To do this, we will isolate genomic DNA from the zebrafish (week 2), amplify just one region of the *lin28a* gene that will or will not contain the mutation using a technique called **polymerase chain reaction (PCR)** (week 3), digest the amplified DNA with the restriction enzyme KpnI, and see the different sizes of DNA using agarose gel electrophoresis (week 4). More information on PCR and agarose gel electrophoresis is below.

2. *If your fish has a WT gene, will KpnI cut the gene?*

3. *If your fish has a mutant gene, will KpnI cut the gene?*

I. Molecular Biology Skills

When performing molecular biology experiments, you will be using extremely small volumes of solutions. Therefore, you will need to become comfortable estimating and working with micropipets to ensure accuracy. You must complete this section before moving on to the fish surgery.

Appendix 2. Student Lab Manual

Pipetting small volumes

Micropipets are the main tools of any molecular biologist. Compare the four sizes of micropipets: P1000, P200, P20, and P2. The volume limits of each micropipet are different and can be found on the round top part of the micropipet. A P1000 pipets from 100 µL to 1 mL, a P200 from 20 µL to 200 µL, a P20 from 2 µL to 20 µL, and a P2 from 0.1 µL to 2 µL so they are named after the highest number they can pipet. (NB: Although the pipets can be set higher or lower than these volumes, it is never recommended for accurate pipetting.) To set a micropipet to a specific volume, it should read like this:

	P1000	**P200**	**P20**	**P2**
Top:	0	2	0	2
	2	0	2	0
Bottom:	0	0	0	0

In this case, the P1000 and the P200 are both set to 200 µL. The P20 and P2, however, are set to 2 µL. For the P20, the bottom number is the decimal place, and for the P2, the bottom two numbers are decimal places.

To change the settings on a micropipet, rotate the wheel at the top until the numbers read the desired amount. *Demonstrate that you understand by setting the appropriate pipets to 40µL and 10µL. Show your instructor before moving on.*

Before pipetting, the micropipet requires a disposable plastic tip. A P1000 uses a blue tip, P200 and P20's use a yellow tip, and P2's use small, clear tips. Use the pipet to tap the appropriate tip onto the end. Your tips are sterile so once a tip is on the micropipet, do not allow it to touch *anything* but the liquid you would like to pipet and the container you are pipetting into.

To use a micropipet, hold the pipet upright in your dominant hand. Put a tip on the micropipet. Depress the plunger to the first (soft) stop before going into the liquid. With your thumb down, submerge the plastic tip in the liquid to pick up the solution. To move the liquid somewhere else, depress the plunger gently to the second (hard) stop to release the liquid. Make sure that once you have liquid in the pipet that you keep it upright, ie ***the tip should NEVER be level with or above the handle.***

Write the steps for using a micropipet in your lab notebook in your own words.

Procedure #1: Practice micropipetting

Each person should obtain a tube of blue dye. Before starting, make sure all of the dye is at the bottom of the tube and not on the sides; if not, tell your instructor. Set the appropriate micropipet to 10 µL. Check with your instructor if you are unsure. Lay a rectangle of parafilm flat on the bench. How many 10 µL-sized dots can you pipet onto the parafilm? Check with your instructor before moving on.

Procedure #2: Survival Surgery and Genomic DNA Isolation

Because this is a sensitive process, *only two fish will be put to sleep at any point*. We do not want them to be anesthetized for too long and die. Please make sure you and your partner have read the procedure and are prepared before notifying your instructor that you are ready.

1. Get a pair of gloves that are light colored (dark gloves make this more difficult).

2. Label a 0.5 mL tube with your initials and the date and the name of your fish. (Note: write directly on the tube; do not use the tape.)

3. Clean your scissors with ethanol and wipe them down with the kimwipe. Leave them sitting on a kimwipe on your bench.

4. While one partner is timing the procedure, using the net, put one fish into the tank with the anesthetic (MS-222, also known as tricaine). Within three minutes, the fish should stop swimming.

5. Once the fish has stopped swimming, use the net to gently lay the fish in the open petri dish on your bench. Carefully cut the tail fin, making sure not to cut into the body (Figure 4). Move the fin immediately into the appropriate tube. Use the forceps if you need them.

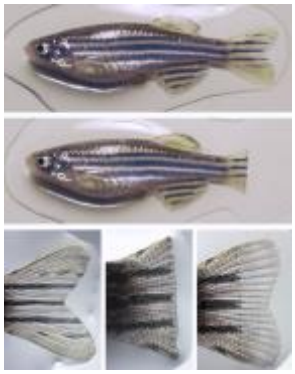


Figure 4. Example of where to cut zebrafish tail (http://www.entwicklungsbiologie.uni-bayreuth.de/en/research/index.html).

6. Place the fish into a new tank and name the fish by putting a piece of tape on the tank with the fish's name. *Make sure the name is written in your notebook and that it matches the name on the tube.*

7. Add 10 µL 50 mM NaOH to the fin. Using the micropipet tip, push the fin down into the solution if it is on the side of the tube.

8. Put the tube with the fin and NaOH into the 95°C heat block for 20 minutes. After the incubation, the fin should have dissolved and the solution may look greyish or green.

9. Add 2.5 µL 1 M Tris-HCl, pH 8 to the fin to neutralize the sodium hydroxide.

10. Add 40 µL water to the tube.

11. Make sure the tube is still clearly labeled and put it in the ice bucket at the front of the room. Next week, you will use this as a template for a PCR.

Appendix 2. Student Lab Manual

Week Three: Genotyping Zebrafish Part Two

Background

Today, we have two things to accomplish. First, in order to tell if *lin28a* is regulating other genes involved in regenerating the zebrafish tail fin, we will isolate tissue from the partially regenerated tail as we did last week. As soon as this tissue is in a tube, we will flash freeze it in liquid nitrogen so that we will be able to isolate mRNA later in the semester. This mRNA will tell us which genes have been turned on and how high (changes in gene expression).

If lin28a *is necessary for upregulating the genes (turning them on) involved in regeneration, how would you expect the gene expression pattern to differ between WT and mutant fish?*

The second thing to do is to set up a **Polymerase Chain Reaction (PCR)** using the DNA you isolated last week from the tail fin. This PCR will amplify a specific region of the *lin28a* gene using primers specific to only that gene. The portion of the gene you amplify should contain the region that is deleted in mutant fish. While in theory we could try to cut the gene directly from the genomic DNA, genomic DNA is huge, with millions of base pairs per chromosome. Doing this would be extremely unwieldy and impossible to distinguish just the *lin28a* gene. However, by amplifying the region using PCR first, we target only the DNA region we are interested in (approximately 750 base pairs). Furthermore, because PCR results in thousands of copies of a specific region, we will have much more DNA of that region to examine. We will use KpnI to cut this region of the lin28a gene next week. Depending on whether your fish contains a WT or mutant gene, the DNA will cut to different sizes, which can be detected by running the DNA on an agarose gel next week. Though setting up a PCR is a technically simple process, there's a lot going on in the PCR tube, and you should complete all the analysis before leaving lab today.

PCR is a simple technique which is used in a whole range of applications from identifying viruses, to crime scene analysis, to diagnosing diseases, to identifying the illegally caught fish in fish markets, to sequencing entire genomes. It is one of the most important biological techniques ever developed. The process mimics DNA replication by utilizing the enzyme DNA polymerase. The PCR reaction is performed in very small tubes with special thin walls to allow the temperature to change in the solutions inside the tube very quickly. The machine crucial to this reaction is called a **thermocycler**. It is basically a machine which heats and cools the tubes for various lengths of time as needed. The important components of a PCR reaction are as follows:

1. ***Taq* DNA polymerase**: A version of polymerase taken from thermophilic bacteria. It is extremely heat-tolerant, which is important to this procedure.
2. **DNA template**: Fish genomic DNA you isolated last week. Because PCR makes thousands of copies of DNA, you only need a tiny amount of DNA to start.
3. **Primers**: Short, single stranded pieces of DNA which are complementary to each end of the DNA region being amplified. Primers specifically bind to the complementary DNA using hydrogen bonding and allow the polymerase to start adding base pairs, just like in DNA replication.
4. **dNTPs**: A mixture of adenine, guanine, cytosine, and thymine deoxyribonucleotides. These are the building blocks of DNA and serve as the raw material as the DNA strands are built by the DNA polymerase, one base at a time.

5. **Buffer**: Contains salts and magnesium ions needed to create the proper pH and ion environment for the reactions to occur.

The process of PCR involves many cycles; see the figure below. Each cycle has the following steps:

1. **Denaturation step**: The reaction is heated to 95ºC to allow DNA strands to unwind and separate along the hydrogen bonds that are responsible for base pairing.
2. **Annealing step**: The reaction is cooled to 55ºC to allow the primers to stick to complementary regions of the template DNA, setting the stage for the DNA polymerase. (This temperature is too cold for *Taq* DNA polymerase to function.)
3. **Extension step**: The reaction is heated to 68ºC, a temperature where DNA stays bound together with the primers, but the *Taq* DNA polymerase enzyme is fully functional. The enzyme starts at the 3' end of the primer and fills in bases complementary to the template strand moving from 5' to 3'.

These three steps (1 cycle) are repeated 30 times. Each time the cycle is repeated, the number of copies grows exponentially until you have millions of copies.

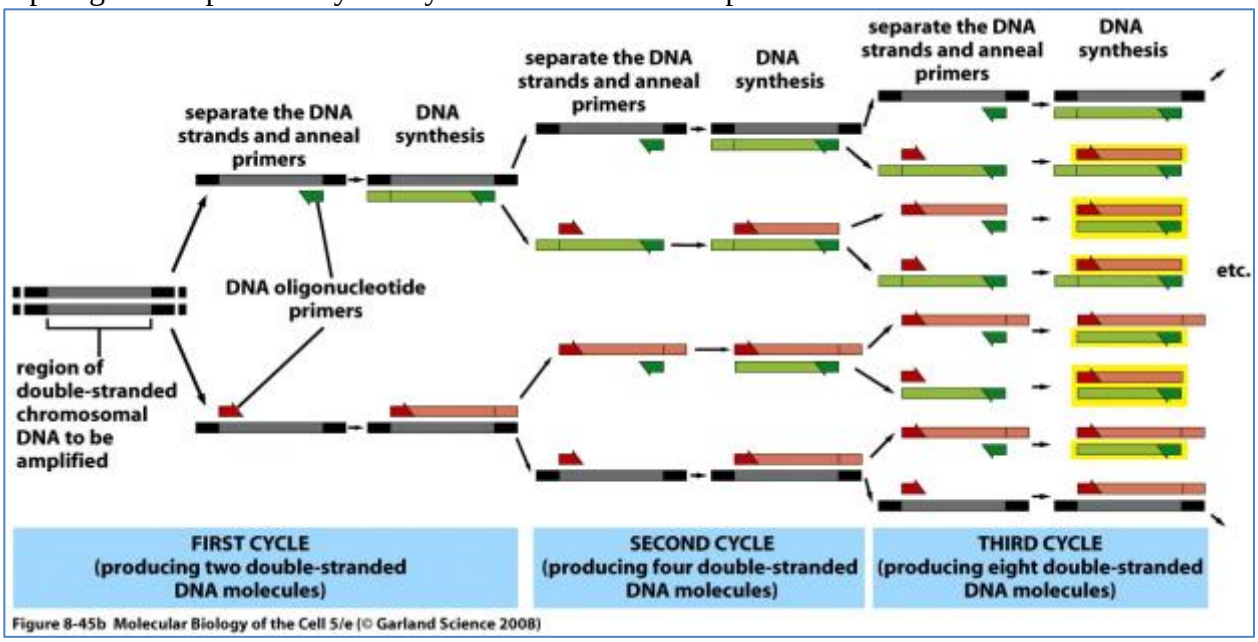


Figure 8-45b Molecular Biology of the Cell 5/e (© Garland Science 2008)

The laboratory procedures do not need to performed in order, and you should work on answering the questions under predictions for analysis while you wait.

Procedure #1: Survival Surgery for mRNA Isolation

Before beginning this procedure, note the appearance of the regenerating tail in your lab notebook. What color is it? How big?

Because this is a sensitive process, *only two fish will be put to sleep at any point*. We do not want them to be anesthetized for too long and die. Please make sure you and your partner have read the procedure and are prepared before notifying your instructor that you are ready.

1. Get a pair of gloves that are light colored (dark gloves make this more difficult).

2. Label a 0.5 mL tube with your initials and the date and the name of your fish.

3. Clean your scissors with ethanol and wipe them down with the kimwipe. Leave them sitting on a kimwipe on your bench. Alternatively, you may use a razor for this process. Clean your scissors in between each fish or use a different razor for each fish. Make sure the razors are properly disposed of in the sharps waste.

4. While one partner is timing the procedure, using the net, put one fish into the tank with the anesthetic (MS-222, also known as tricaine). Within three minutes, the fish should stop swimming.

5. Once the fish has stopped swimming, use the net to gently lay it in the open petri dish on your bench. Carefully cut the tail fin, making sure not to cut into the body. Move the fin immediately into an appropriately labeled tube. Use the forceps if you need them.

6. Place the fish into a new tank to recover. *Make sure the name is written in your notebook and that it matches the name on the tube.*

7. Put the tube in the liquid nitrogen at the back of the room. Once all the tubes are in the liquid nitrogen, I will store them at -80°C until we are ready to isolate mRNA.

Procedure #2: PCR from Genomic DNA for *lin28a*

In order to set up a PCR reaction, I have created a **master mix cocktail** that contains all the chemicals necessary for a PCR. It should be on ice whenever someone is not actively using it.

In your lab notebook, make a chart of everything that is in the master mix with the concentrations. Hint: see the background for the components and this website for the precise concentrations (https://www.neb.com/protocols/1/01/01/protocol-for-a-routine-taq-pcr-reaction).

For each DNA sample, put 23 µL master mix into a PCR tube. Add 2 µL genomic DNA to the PCR tube. Make sure that all the solution is at the bottom of the tube and the tubes are clearly labeled. Put the tubes in the PCR rack in the ice bucket at the back of the room. Once everyone has completed this, I will take the PCR tubes to run in the thermocycler. Make sure all your DNAs get put away in the freezer box.

The PCR will be cycled as follows. *Include this chart in your notebook as part of your procedure.*

Cycle Step	Temperature	Time	# of Cycles
Initial Denaturation	95°C	30 seconds	1
Denaturation Annealing Extension	95°C 55°C 68°C	15 seconds 45 seconds 1 minute	30
Final Extension	72°C	5 minutes	1
Hold	4°C	indefinitely	1

1. *What do you think is the purpose of the final extension?*

2. In PCR, we use a polymerase enzyme that works best at a high temperature (68-72°C) and is not denatured at 95°C. *What do you think would happen if we used a human DNA polymerase enzyme? Would it work for PCR? Why or why not?*

Analysis: What will be happening in that PCR tube? Watch the video here: http://www.hhmi.org/biointeractive/polymerase-chain-reaction-pcr. Complete the following questions in your notebook to help you predict the products of your PCR.

3. *Create the following chart in your notebook to draw what your DNA looks like at each step of a PCR. Include the primers.*

Temperature	Image
DNA at room temperature	
DNA at 95°C	
DNA at 55°C	
DNA at 68°C	

For each PCR reaction, you need a forward primer (F) that starts at the 5' end of the gene and a reverse primer (R) that starts at the 3' end of the gene. The primers we are using for this experiment are as follows:

lin28a.gDNA.F: 5'-CTTCATATGGAGGGTTTTCGCAGTC
lin28a.gDNA.R: 5'-AGGACCACCACAACAAATGCAG

You and your partner should determine where these primers are in the zebrafish genome. To do so, you'll need to go to the BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Click the link and read the top of the website.

4. *In your own words, what is the function of this freeware program?*

Click on Nucleotide BLAST. Copy/paste the forward primer sequence into the text box under "enter accession number(s), gi(s), or FASTA sequence(s)".

5. *What is the full scientific name for zebrafish?*

Scroll down to the "Organism" box. Type in Danio (taxid:7954). Click the blue BLAST button at the bottom left. Once the program is done processing, you should see a "Graphic Summary". Your input primer sequence is the query, and the lines below it represent matches among all the

zebrafish sequences ever entered into this database.

6. *Recall from two weeks ago, on which chromosome is* lin28a*?*

If you scroll down to the "Descriptions" box (or mouse over the lines that cover the sequence in the graphic summary), you should find some sequences that refer to a **linkage group**. In this case, linkage groups are another way to say chromosomes, as in all the genes on a chromosome are linked together, much like all the pearls on a single necklace would be linked together. Click on the link that matches the chromosome number indicated in question 6. The "Sbjct" line is the part of the linkage group that matches your primers, and the numbers adjacent to the sequence represent the specific location where your primer aligns (matches).

7. *Write the left number down for the sbjct line.*

8. Repeat the BLAST analysis for the reverse primer, and *record the left number.*

9. The difference between these two numbers represents the size of the piece of DNA that your primers should amplify during the PCR. *What is the size of the DNA amplified by your PCR?*

For your convenience, I have pasted the nucleotide sequence below. Although only one sequence is shown, recall that this DNA is double-stranded, and the complementary sequence is not indicated. In addition, each line reads into the next so that it is a continuous strand. Spaces are included merely to make the sequence easier to read. They do not exist in the chromosome.

agcaagcttc atatggaggg ttttcgcagt ctgaaggaag gagaagcagt ggaattcacc
tttaaaagat cctctaaggg tctggagtcc ttacaagtga cggggcctgg aggggcacca
tgtgtgggca gcgagaagaa acctaagggt acccaaaaga ggcggtcaaa aggagatcgg
tgagtgtctt gctctgttgc tgctatttta ctgtttatt catttgatca atcagagttt
ttccgaaatg ctggcccatt ttccagatct tatattaggt taatgcagat atacaatata
tgtacatcct tttacagaaa gcgctctaaa acgtatttca gtcagtgtag cagtatatac
gctcaaacat ttatttttga aatgcgttca aactaattta aaatgagcta aatcaataat
tcctgaggtt taaaatgaat agtttatgt tcaattcact taaaacttta aaacaatcaa
gttaacttaa tcaatttgtg ttggggcagc atgaaggaac tgtgaggaac ccagcatttt
tttttttat ttttttttac aatgcaacaa tcatcttaca ttttcaacaa gaatcattta
atgatattaa aagaccctgt ttaaagtcca tgtgaaatta atcatgttta ttttgtgcaa
gttaatccac aaaaaaaaaa aaaaaaaacg taatcttgtt gttttttgg tttggtgat
cttcaaagtc tgaccatttg cttctgcatt tgttgtggtg gtccttctct gttgacatca

Underline or highlight the sequence of the forward primer. (Hint: this is near the beginning.)

Finding the sequence of the reverse primer is a bit trickier because you will need to find the **reverse complement** of the primer sequence since the reverse primer is identical to that strand of DNA and not the one shown. To do this, first answer the following questions.

10. *Which base is complementary to A?*

11. *Which base is complementary to T?*

12. *Which base is complementary to G?*

13. *Which base is complementary to C?*

14. *Based on your answers, what is the complementary sequence of the reverse primer? Include the annotation 3' at the beginning.*

15. DNA strands are antiparallel with a 5' end near the 3' end of the complementary strand and vice versa so now *write out the reverse complement of the reverse primer. (Hint: this just means write the complementary sequence backwards.) Include the annotation 5' at the beginning.*

Using the reverse complement of the reverse primer, underline or highlight this region of DNA on the sequence. (Hint: this is near the end.)

The last thing to do with this sequence is to show where the KpnI site is and predict the sizes of this wild-type PCR product after a digestion with KpnI. Although you could just read the entire sequence until you find a KpnI site (GGTACC), fortunately, there is freeware, called NEBcutter (http://nc2.neb.com/NEBcutter2/), that will do that for us. Copy and paste the sequence from 5'-primer end to 3'-primer end into the large textbox on the NEBcutter website. Click on the submit button to the right of the box. NEBcutter will generate a graphic of the inputted sequence as a line with the 5' end representing number 1, the 3' end at the right, and all the restriction enzymes that cut the sequence below the line. When you hold your mouse over a restriction site, it will tell you the precise nucleotide number counting from the 5' end that is cut. Hold your mouse over the KpnI site.

16. *What number is indicated?*

17. *If you cut the PCR product with KpnI, what are the sizes of DNA fragments you will generate?*

18. *In your notebook, define the following terms: heterozygous, homozygous, allele, and diploid.*

Appendix 2. Student Lab Manual

Week Four: Genotyping Zebrafish Part Three

Background

Today, we will finally see the results of your experiments and hopefully determine the genotype of your fish. First, we will digest half of your PCR with KpnI, and then we will examine the digested and undigested PCR products using agarose gel electrophoresis. You will then be able to determine whether your fish is a **homozygote** or a **heterozygote** for the *lin28a* gene.

In order to characterize DNA restriction fragments, to verify that restriction digests have worked, or to purify restriction fragments, it is necessary to separate and identify the various sizes. The most common method to do so is **DNA agarose gel electrophoresis**. With the consistency of Jello, agarose is actually a molecular sieve filled with pores. Small fragments of the DNA can move quickly through the pores, while large pieces take more time. DNA has too little mass to separate by gravity; instead, an electrical current is applied to the gel to force the DNA in one direction. The phosphate groups in the DNA backbone are negatively charged, and therefore, the DNA will only move toward the positive electrode. By comparison to a set of DNA molecular weight standards (or ladder) loaded in the same gel, it is possible to accurately determine the size of DNA fragments in a sample of unknown composition.

Linear DNA in an agarose gel migrates at a rate that is inversely proportional to the log of its molecular weight. When dealing with nucleic acids, size in number of base pairs is often substituted for molecular weight. Here is a link to an image of a 100 bp DNA ladder with the molecular weight (in base pairs) of each band, or DNA fragment: https://www.neb.com/products/n3231-100-bp-dna-ladder. The 1 kb and 500/517 bp bands are always the brightest in this particular molecular weight marker, and therefore, they can be used for orientation. You should be able to determine the size of your DNA fragments by comparing them to the size of the standards in the ladder.

DNA is obviously not colored, and so we use an orange dye called ethidium bromide to stain the DNA. Ethidium bromide becomes fluorescent after excitation with UV light so that we can see the location of the DNA. Ethidium bromide is considered a carcinogen. Though it does not directly mutate DNA, it does insert between the DNA base pairs.

Procedure #1: Restriction Digestion

1. For each PCR, you will perform one digest with KpnI and have one control of undigested DNA. Label your 0.5 mL tubes clearly.
2. Finish the buffer calculations for the chart to the right. You have 10X Buffer, but you want 1X Buffer in a total volume of 20 µL. (Hint: Use $C_1V_1=C_2V_2$.)
3. Write the water volume in the chart by bringing the remaining volume up to 20 µL. (water volume = total – 10 µL DNA - ____µL 10X Buffer - ____µL restriction enzyme). Double check your numbers with your professor before proceeding.

all volumes in microliters (µL)	KpnI	undigested
PCR	10	10
10X Buffer		
Water		
KpnI RE	1	0
Total	20	20

4. Each group should set up the restriction digests by combining the components in the column in the appropriate tube. ***Keep the enzymes on ice, and always add the enzymes last.*** (Hint: Since you are using small volumes, it helps to check off the items as you add them so that you know what is already in the tube and what is yet to be added.)
5. Gently mix the reactions by tapping them.
6. Shake the liquid to the bottom of the tube by flicking your wrist.
7. Put the reaction in the 37°C water bath for at least 30 minutes.

Procedure #2: DNA agarose gel electrophoresis

1. Add the appropriate amount of 6X loading dye (____μL) to your restriction digest and undigested DNA to have a final volume at 1X. Once the dye is added, you now have samples that are ready to load.
2. Load 15 μL of your sample into the 2% agarose gel as shown in figure 5. Since several groups will use the same gel, make note of where your samples are and their order in the gel on the board.
3. Once all samples and a molecular weight ladder are loaded, put the lid on the gel, and plug it into the power source, making sure that the DNA is running toward the cathode (positive).
4. Run the gel at 150 volts for approximately 1 hour or until the blue dye has run at least 3/4 of the way down the gel.
5. Image the gel using the UV transilluminator. Your instructor will post the electronic files so that you can include a labeled figure in your lab report.

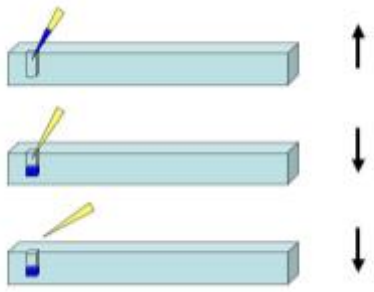
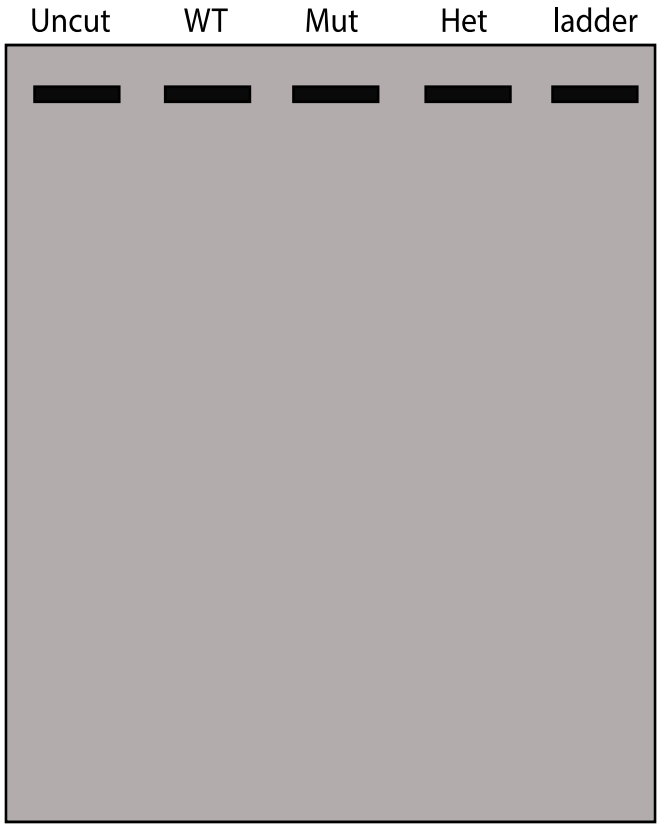


Figure 5. How to load an agarose gel. Once your sample is in the tip, submerge the tip in the buffer and put it gently into the well. Push the sample out of the tip (go until all the blue is out of the tip; you may only have to go down to the soft stop). Keep your thumb down until you remove the tip from the liquid. Otherwise, you will suck water back up into the tip. The arrows on the right indicate the direction of the plunger at each point.

Predictions for Analysis

While you are waiting during this lab, complete the following in your notebook. Hint: you will need your notes from the previous weeks to do this exercise.

Draw a gel template like the one shown below. The black lines at the top represent the wells where you loaded your DNA.



First, draw in the DNA bands (lines) of the 100 bp molecular weight standard or ladder. On the right side (next to, but not on the gel), indicate the sizes of the bands in bp.

Using the ladder, draw a single band at the appropriate molecular weight under the well for uncut.

If your fish has only WT lin28a *genes, will they be cut by KpnI? Draw the appropriate band(s) under the WT lane. Note: If two bands are the same size, they will appear as one since they migrate the same distance on the gel; they are overlaid.*

If your fish has two mutant lin28a genes, will they be cut by KpnI? Draw the appropriate band(s) under the Mut lane.

What if your fish has one WT lin28a gene and one mutant lin28a gene? Draw the appropriate band(s) under the Het lane.

You have now generated an answer key to which you can compare your own data.

Results and Conclusions

Include a fully labeled picture of your gel in your notebook. Note: It is not appropriate to draw on or circle your data. If you feel you must indicate specific bands of interest, you can draw an arrow or place an asterisk *next to* the bands.

What are the genotypes of your fish?

You will have to generate an electronically labeled figure with a figure legend to be submitted. You may create the figure in powerpoint or Adobe software.

Appendix 2. Student Lab Manual

Week Five: Destroying Zebrafish Hair Cells with Copper Sulfate

Experimental Design: Complete this section before lab and before reading ahead.
For this lab, we will use copper sulfate ($CuSO_4$) to destroy the hair cells in the neuromasts in zebrafish larvae that are 5 days post fertilization (dpf) old. In wild-type zebrafish, these cells should regenerate completely within 2 days of treatment. Next lab, we will do alkaline phosphatase staining to visualize the neuromasts (see Figure 1 from Lush and Piotrowski, 2014).

The goal of this experiment is to examine the difference in the rate and/or capacity of *lin28a* mutant larvae to regenerate hair cells as compared to wild-type zebrafish. With your partner, design an experiment to test this idea. Answer the following questions to assist you in your experimental design.

1. *What would you expect to see if the hair cells cannot regenerate in 7 dpf* lin28a *mutants as compared to wild-type hair cellss, aka what is your hypothesis?*

2. *What is your experimental treatment condition to answer this question? Which fish will you use, and what will you do to them?*

3. *What will your negative controls be, and what are they controlling for? (Notice this is plural.)*
   Hints: Can you think of a situation where the treatment conditions are the same, but you get a known result? How do you know that the hair cells are present at all in mutant embryos?

4. *Other things to think about:*
   What could you do to "know" that the hair cells were destroyed by the copper sulfate?

   How big do you think your sample size should be for each condition? As in, would you treat just one larva, or more than one?

5. *Make a flowchart of your experimental design in your notebook to show your instructor before starting the experiment.*

Procedure

1. Prepare your solutions. Show your math in your notebook, and check with your instructor before proceeding.

    a. You will need 200 mL of 1X Holtfreter's Buffer (1X HB). 1X HB consists of 60 mM NaCl, 0.9 mM $CaCl_2$, 0.67 mM KCl, and 10 mM HEPES. We have provided a 20X HB solution, and you will need to make 200 mL 1X HB. *How much 20X HB, and how much water will you need?* (Hint: Use $C_1V_1 = C_2V_2$)

    b. You will need to make a working solution of copper sulfate by doing a 1:100 dilution of 10mM $CuSO_4$ into a final volume of 15mL in 1X HB. *What volume of 10mM $CuSO_4$ will you need for 15mL? What is the final concentration of the $CuSO_4$?* (Hint: When diluting a solution by a dilution factor, the dilution factor does not have any units. To solve this, first represent your dilution factor as a fraction; for example, 1:10 would be 1/10. Then you can multiply your final volume by the dilution factor to get a volume of your concentrated stock solution. You can do the same thing for the concentration.)

2. Distribute your buffers to various plates and label the plates as appropriate.
    a. You will need one small petri plate with diluted $CuSO_4$ in 1X HB. Pour the solution into the plate, and cover the lid of the plate with foil so that the lid will still fit on the plate.

    b. You will need eight large petri plates of 1X HB.

    c. You will need one large petri plate of 1X E3.

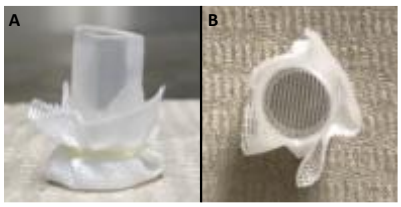


Figure 6. Fish strainer construction. A represents a side view, and B is the view of the bottom.

3. You will need one fish strainer (Figure 6) for ***each treated sample***. Label them as appropriate. Submerge the netted bottom of the fish strainers in the dish filled with the E3 buffer so that the open end is facing up.

4. Using a plastic transfer pipet, collect 10 to 20 – 5 dpf larvae to put in the appropriate fish strainer. Pick larvae that look healthy and have their swim bladders. (These are the shiny silver bubbles in the middle of the larvae; see figure 7.)

5. Once the larvae are in the strainer in the E3, transfer the strainer with larvae to a dish with 1X HB. Incubate for two to five minutes in this buffer.

6. Using tweezers, pick up the strainers and **gently** dab the excess HB on a paper towel before placing them

Figure 7. The red arrow indicates the swim bladder in a 5 dpf embryo. Image from https://www.sanger.ac.uk/sanger/Zebrafish_Zmpgene/ENSDARG00000016153.

into the diluted $CuSO_4$ solution. Gently cover with foil.

7. Incubate larvae in diluted $CuSO_4$ for 1 hour. Meanwhile, collect your untreated larvae and place them into a large petri dish of 1X HB. Use masking tape to ensure that each plate is **clearly labeled** with all the relevant information about the type of fish, the treatment (or lack thereof), the date, and your initials.

8. After 1 hour, use tweezers to pick up the strainers from the diluted $CuSO_4$ solution and place them into a new dish of 1X HB.

9. Move the strainers to another dish of 1X HB for 5 minutes.

10. Repeat step 9.

11. Using a transfer pipet, suck up 1X HB from a new dish and squirt the larvae out of the strainer and into the plate by holding the strainer sideways so that the net is facing you. Make sure to label and use a different plate for each sample.

12. Carefully move all your plates into the bin so that the larvae can be moved to a 28.5°C incubator. The larvae will incubate there for 2 days to recover and *possibly* regenerate.

13. **In two days, you will have to fix your larvae. Wear appropriate lab attire and gloves for this process. Both partners do not have to be there, but decide who will have to come in (at least one from each pair).** To fix your larvae, collect the larvae in a 1.5mL tube using a transfer pipet. Remove as much liquid as possible without removing the larvae. Add 1 mL of 4% paraformaldehyde/PBS to the tube. Make sure the tubes are clearly labeled with the sample description (or a secret code that will be written and defined in your notebook), the date, and your initials. Store the larvae in the refrigerator until the following laboratory.

Appendix 2. Student Lab Manual

Week Six: Visualizing Neuromasts using Alkaline Phosphatase

Background

Today we will use the fish larvae you collected last week to determine if they have regenerated their hair cells. The zebrafish produces the enzyme alkaline phosphatase in the neuromasts (Gilmour et al., 2004; Lush and Piotrowski, 2014a, 2014b; Villablanca et al., 2006). Therefore, we can use a substrate of alkaline phosphatase, nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP), to visualize where alkaline phosphatase is active. When this substrate is added, alkaline phosphatase converts it to a dark blue-violet color. For those of you in organic chemistry, the reaction is shown to the right if you're curious (Figure 8). This procedure will allow us to identify both the number of neuromasts and their size in your treated and untreated fish. A direct comparison with your controls should tell us whether the hair cells have regenerated fully.

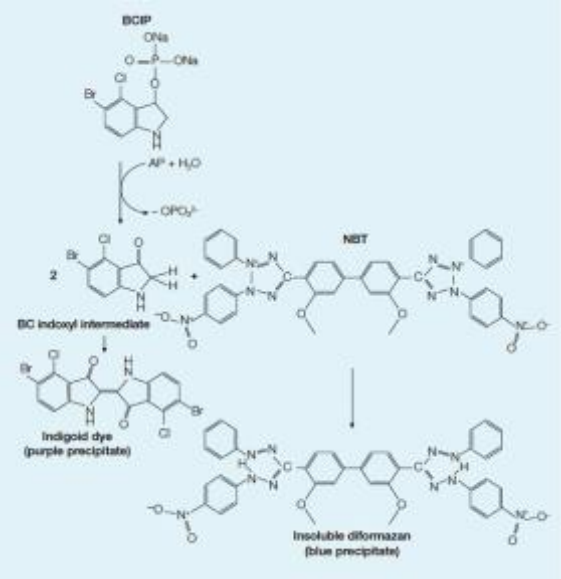


Figure 8. BCIP and NBT react to form a dark blue-violet precipitate in the presence of alkaline phosphatase. (http://www.bio-rad.com/en-us/applications-technologies/detection-methods#2)

1. *Based on the samples you prepared last week, make a chart in your notebook about your predictions. Save a column that will include your actual results. Do you expect to see neuromasts of a 'normal size and number' in each sample? Which sample is your control that will define 'normal size and number'?*

2. *For an enzyme named alkaline phosphatase, what sort of conditions do you think it will require to work? Hint: what do you think distinguishes the staining buffer from PBS? Why will the PBS stop the reaction?*

Procedure

Notes: Wear gloves for this entire procedure. You should be careful to not to suck up your larvae so hold the tube up so that you can see what you're doing while you are removing solutions. You should not suck the larvae dry but leave approximately 100 µL left in the tube every time you remove a solution. All solutions except the paraformaldehyde can go into the waste beaker.

1. Remove most of the paraformaldehyde solution from your larvae using a micropipet and pipet the solution into the designated paraformaldehyde waste container.

2. Wash the larvae with phosphate buffered saline (PBS)/0.3% Tween-20 for 5 minutes by adding approximately 1 mL PBS/0.3% Tween-20. Invert the tubes several times.

3. After the 5 minutes, remove the solution into a beaker using a micropipet and add another mL of PBS/0.3% Tween-20. Incubate 5 minutes. Invert the tubes several times.

4. Repeat step 3 once more.

5. Remove the PBS/0.3% Tween-20 solution into the beaker and wash the larvae with 1mL staining buffer (50 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris pH 9.5, 0.1% Tween-20) for 5 minutes. Invert the tubes several times.

6. After the 5 minutes, remove the solution into a beaker using a micropipet and add another mL of staining buffer. Incubate 5 minutes. Invert the tubes several times.

7. Repeat step 6 once more.

8. Add 2.25 µl NBT and 5.75 µl BCIP to 1 mL of staining buffer (this staining solution will be prepared for you). Add 500 µl staining solution to each tube. Cover the tubes in foil. Let sit at room temperature for 10 minutes then check to see if the neuromasts are stained by looking through the microscope. If not, let sit longer and check periodically until they are stained.

9. Once the embryos are a desired intensity, all samples should be stopped by removing the staining solution and adding 1 mL PBS. Invert several times, remove the PBS, and replace with an additional 1 mL PBS.

10. Place the embryos in a well of a white porcelain spot plate so that they can be imaged, and the neuromasts counted and measured.

In addition to recording your results of the count and size of the neuromasts for at least 3 fish per sample, you will have to take pictures of a representative sample of a fish in each condition. The quantitative data should be presented in graphical form. A full figure with both pictures and graphs should be submitted by the beginning of the next lab. Hint: It will have panels A-F.

Week Seven: Measuring Changes in Gene Expression
Part One: RNA Isolation

Background

The **phenotype** of your cells is dictated by which proteins are expressed, or made, in every cell. Though the cells in your body contain the same DNA, different cells express different proteins, giving them a different function. For example, you know that the cells in your brain must perform different functions than those of your muscles or of your skin. This distinction is mediated by cells having different amounts of proteins and/or different proteins altogether.

In the case of our zebrafish, we would like to examine changes in expression in the absence of the *lin28a* gene during regeneration. After injury, not only is *lin28a* increased in expression, but *lin28a* has been reported to affect transcription of *c-myc*$_b$ (Ramachandran et al., 2010); refer to figures 1a-c.

1. *Examine the figures in the paper. What conclusions did the authors make about* lin28a *and* c-myc$_b$*?*

2. *What is a pluripotency factor?*

Typically, we can measure a change in gene expression by measuring the amount of proteins using a technique called a western blot. Essentially, this method can tell you how much of any particular protein you have in a cell or tissue. However, it requires the use of specialized proteins called antibodies. Antibodies are produced by an animal's immune system and are used to defend the animal against the myriad of pathogens the animal may encounter. Because there are so many different pathogens, antibodies are highly specific to the protein of interest, the antigen. Although there are many different antibodies commercially available for human proteins, there are fewer for zebrafish and other model systems. Therefore, we will have to use an indirect measure of gene expression by examining changes in mRNA. From this mRNA, we will use an enzyme called **reverse transcriptase** to make complementary DNA, or **cDNA** (Figure 9)**.** Then we can perform PCR for the gene of interest using the cDNA as a template. This will tell us whether the mRNA for that particular gene was present, and ideally, approximately how much of that mRNA was present. This process (isolating mRNA, converting it to cDNA, and doing PCR)

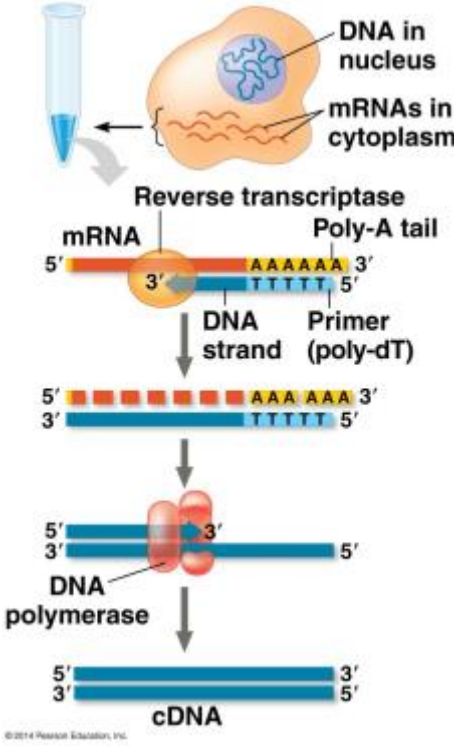


Figure 9. mRNA is used as a template for reverse transcriptase to generate complementary DNA.

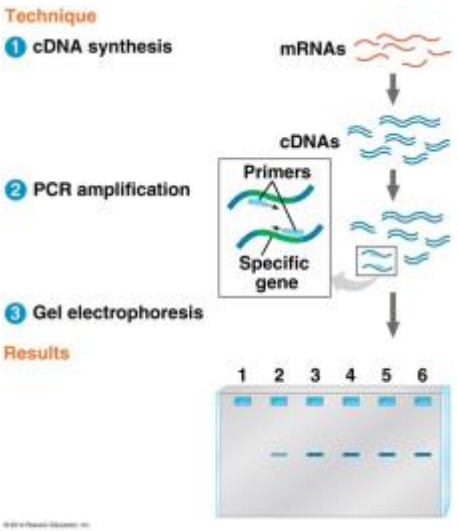


Figure 10. cDNA can be used as a template for PCR to measure differences in gene expression among samples (numbered 1 through 6 here).

is called reverse transcription PCR, or **RT-PCR** (Figure 10).

3. *How will the amount of mRNA tell us about how much protein is made? What is the relationship we can infer between these two types of biological molecules?*

4. *If a cell does not express the gene, will you detect a signal by RT-PCR? Explain.*

5. *How is cDNA distinguished from genomic DNA?*

Remember that second tail we isolated and dropped into liquid nitrogen? Now is the time to use it! Because you isolated it seven days after your first surgery and it takes a zebrafish approximately fourteen days to fully regenerate a tail, we can assume that the tissue you isolated is in the process of regeneration. Today, we will isolate the RNA and store that in the freezer until we are ready to do the PCR and electrophoresis.

Procedure

Note: this procedure is adapted from the Qiagen RNeasy Mini Kit (https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en; see the picture on page 9 of this handbook for an overview).

1. Wear gloves for this whole procedure. Your skin is covered in RNases that will degrade your sample.

2. Find your tube containing the tail fragment from the ice bucket.

3. Squirt 600 µL RTL Plus solution onto the tail tissue.

4. Using the 1 mL syringe, suck the solution up and squirt it out at least ten times. This breaks up your tail so that it can be dissolved by the solution. Be careful not to spill the solution by pushing out too hard.

5. Spin 3 min full speed.

6. Carefully remove the liquid using a P1000 micropipet and put the liquid in a gDNA Eliminator column. The pellet may not stick so be careful to avoid it. This step allows the genomic DNA to stick to the column, and the RNA (what we want) will end up in the flow-through, or liquid below the column.

7. Spin the column 30 seconds at approximately 9000xg (g represents gravity).

8. Move the flow-through containing the RNA to a new, clearly labeled tube. Discard the column.

9. Add 600 µL 70% ethanol to the flow-through in the new tube. Invert the tube twice.

10. Move 700 μL of the flow-through/ethanol mixture to an RNeasy column. This column will bind to the RNA, and any impurities will flow through the column as waste.

11. Spin the column 15 seconds at approximately 9000xg.

12. Discard the flow-through in the waste beaker. Add the remaining liquid from step 9 into the column, and spin again as in step 11.

13. Discard the flow-through in the waste beaker.

14. Add 700 μL RWI wash buffer. This buffer will wash away any impurities from the RNA bound to the column.

15. Spin the column 15 seconds at approximately 9000xg.

16. Discard the flow-through in the waste beaker.

17. Add 500 μL RWI wash buffer. This different wash buffer will wash away any impurities from the RNA bound to the column.

18. Spin the column 2 minutes at approximately 9000xg.

19. Move the column to a new tube.

20. Spin the column 1 minute at approximately 9000xg. This will completely remove the wash buffers so that we do not have any wash buffer contaminating our RNA.

21. Move the column to a new tube that is clearly labeled with the fish and the date.

22. Add 20 μL pre-heated water to the center of the column. Incubate the tube in the heat block at 55°C for 1 minute. At this high temperature, the water should allow the RNA to dissociate from the column, and the RNA will end up in the flow-through for the next step.

23. Spin the column 1 minute at approximately 9000xg. Discard the column and save the RNA in the ice bucket at the front of the room. This RNA will be stored at -80°C until we are ready to use it.

Appendix 2. Student Lab Manual

Week Eight: Measuring Changes in Gene Expression
Part Two: RT-PCR

Background

In this laboratory, we will use your RNA template to generate cDNA and perform a PCR. We will attempt to confirm or refute some of the RT-PCR data from Ramachandran et al., 2010 figures 1a-c using our *lin28a* CRISPR homozygotes and heterozygotes.

1. *What would be a good control for changes in gene expression after mutation of* lin28a*? In other words, what type of fish could we use to demonstrate the predicted effects of normal levels of* lin28a*?*

2. *In figure 1c, do the levels of actin change? What is the purpose of amplifying actin?*

3. *What would you predict about the amount of Lin28a made by a WT tail during regeneration? Would it be high or low? What about the amount of* lin28a *made by mutant tail during regeneration? Make a chart to describe your predictions for each type of fish and why.*

4. *To your chart, add what you think the effects on* actin *and* c-myc$_b$ *will be in mutant or WT fish and why.*

We can make cDNA and amplify it by PCR all in one tube, provided we add the correct reagents. We will need a separate tube for each gene we amplify.

5. *Which enzyme is used to make cDNA from mRNA?*

6. *Go back to your notes from week three. What will you need in order for DNA replication to occur?*

7. *Which of the reagents listed in your answer to question 2 is specific to the gene you are amplifying?*

Procedure

Note: this procedure is adapted from the One Taq One-Step RT-PCR kit (https://www.neb.com/protocols/2014/08/15/one-step-rt-pcr-protocols-e5315).

1. Wear gloves for this whole procedure. Your skin is covered in RNases that will degrade your sample.

2. Find your tube of RNA from the ice bucket.

3. In order to simplify things, a mastermix containing reaction mix (buffer, dNTPs, dye), water, and enzymes has been made for you. Pipet 9 µL of the green mastermix into one PCR tube per gene you will amplify. In other words, since you will amplify *c-myc$_b$* and *actin*, you should have two tubes per RNA sample.

4. Add 1 μL of the appropriate forward and reverse primer mix to each tube. The primers we are using for this experiment are as follows:

   β-actin-F: 5'- CATCGTTCACAGGAAGTGCTTC
   β-actin-R: 5'- GGTAAACGCTTCTGGAATGAC
   c-mycb.F: 5'- GTCGGTCTGTCGGCTTCCTCCAGGACATG
   c-mycb.R: 5'- CTCTTCTTCATCTTCATCATCTTCGTCATCGGA

5. Add 2.5 μL of your RNA into each reaction. Make sure the tubes are clearly labeled with your initials or fish's name and the gene and place the tubes in the ice bucket at the front of the room. When all the tubes are collected, they will be put into the thermocycler. After amplification, your samples will be stored in the freezer until we can analyze them next week.

6. Before leaving, go to the product website and copy the cycling parameters into your notebook.

Appendix 2. Student Lab Manual

Week Nine: Measuring Changes in Gene Expression
Part Three: DNA Agarose Gel Electrophoresis

Background

In theory, your reactions from last week contain amplified c-mycb and actin DNA fragments. Your goal today is to resolve these fragments on a DNA agarose gel and then determine if mutation of *lin28a* affected the amount of amplified DNA. In order to do this, you will need to normalize the intensity of your *c-myc$_b$* band to your *actin* band.

Procedure

1. Find your PCR tubes in the ice bucket.

2. Load 10 µL of each reaction into a separate well (recall your notes from week four about how to load and run an agarose gel) and run the gel.

3. Image the gel and quantify the intensity of each band. Record these numbers with the genotype of your fish in the Excel sheet.

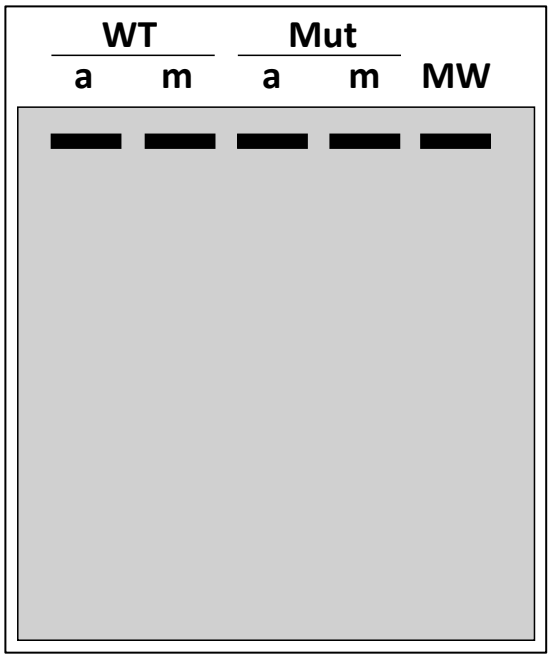
4. *Using class data, generate a graph with error bars and a complete figure legend to describe the effect of lin28a mutation on c-myc$_b$ gene expression.*

Predictions for Analysis

While you are running a gel, you should determine the sizes of the bands that you predict you have amplified using a BLAST search like you did in week three but with the primer sequences for *actin* or *c-myc$_b$* (see last week's lab). Hints: make sure you remember to put in the organism, and you can click on the first gene link you find.

1. *Based on your BLAST search, what is the predicted size of the* actin *DNA fragment?*

2. *Based on your BLAST search, what is the predicted size of the* c-myc$_b$ *DNA fragment?*



Draw a gel template like the one shown below. The black lines at the top represent the wells where you loaded your DNA.
First, draw in the DNA bands (lines) of the 100 bp molecular weight standard or ladder under the MW column (see week four). On the right side (next to, but not on the gel), indicate the sizes of the bands in bp.

Using the ladder, draw a single band at the appropriate molecular weight under the well for the actin *(a) and* c-myc$_b$ *(m) for the wild-type fish (WT).*

Based on your prediction of how lin28a *mutation will affect the expression of both* actin *and* c-myc$_b$*, draw in the appropriate bands under the mutant lanes.*

Appendix 2. Student Lab Manual

Week Ten: Mutation and Behavior

Background

The goal of this experiment is to see how mutation of *lin28a* might affect swimming behavior, specifically **rheotaxis** in larvae. Rheotaxis is dependent on the **lateral line system**. Before coming to lab, you should read the following paper and watch the two movies that are associated with the paper:

Suli, A., Watson, G.M., Rubel, E.W., and Raible, D.W. (2012). Rheotaxis in larval zebrafish is mediated by lateral line mechanosensory hair cells. PLoS One *7*, e29727.

1. *In your own words, define rheotaxis.*

2. *What is the purpose of the lateral line system, and which sensory organs comprise it?*

3. *How have you perturbed the lateral line system of zebrafish larvae in the past?*

4. *Based on your data about neuromasts in* lin28a *mutant fish, do you expect that mutant fish will be deficient in rheotaxis? Justify your answer.*

5. *What are two good controls for this experiment? Which is a positive control, which is a negative control, and why? Generate a chart in your notebook that includes the mutant* lin28a *fish and the two controls and your predictions as to whether rheotaxis will be affected or not.*

Procedure

We will do the experiment shown in Figure 1. Read the "Rheotaxis apparatus and assay" section of the Methods carefully. With your partner, write out your own procedure as to how you will measure rheotaxis. Include the specific times you will use to acclimate the larvae to the chamber, how many pictures you will take, and what times you will take them.

Using class data, generate a graph with error bars and a complete figure legend to represent the rheotaxis among your samples.

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