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miR Seekers: Finding Targets of microRNAs in Undergraduate Lab Courses

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MicroRNAs (miRs) are small, non-coding RNAs that regulate gene expression programs critical for normal development and health. Misregulation of miRs are associated with numerous diseases, and miRs are currently being investigated both as drug targets, and as potential therapeutics. The techniques used to validate miR target predictions are relatively straightforward and inexpensive, making this an ideal vehicle to introduce students in undergraduate labs to authentic research experiences. We describe here a 3-session lab module that we have developed that allows students to generate hypotheses about specific miR candidates, and then design and execute experiments to test their predictions.

Keywords: miR, microRNA, molecular biology, research-based lab

Introduction

Micro RNAs (miRs) are small, 20-22nt RNAs of different sequences, each of which is capable of regulating the expression of several target genes. Estimates of the total number of miRs in the human genome vary widely from the hundreds to the thousands. Whatever the total number of miRs, however, their importance to human health and disease has been clearly established in recent years. miRs have been shown to be important for normal development, and misregulation of miRs have been implicated in numerous diseases – including cancer, mental retardation and obesity. Being central players in human health, miRs are currently being investigated as targets of drugs, and as therapeutic agents. Concurrently, their mechanism of action is also the focus of intense investigation, and we have a pretty good idea of how miRs are generated in the cell, and also how they ultimately regulate the expression of their target genes. However, the exact mechanism by which miRs select their targets is still unclear, and as a result, the algorithms used to predict targets of miRs are somewhat unreliable. All miR target predictions, therefore, require manual validation and testing.

The techniques used to validate miR target predictions are relatively straightforward and inexpensive, making this an ideal vehicle to introduce students in undergraduate labs to authentic research experiences. Students will learn the use of bioinformatics tools, biology databases, and literature searches to select a candidate gene of interest. Along the way, they will be faced with the same questions and decisions that scientists tackle, and students will need to evaluate information from a number of different sources in order to pick their gene of interest. They will then use bioinformatics tools to design primers to test expression of their candidate gene. Finally students will extract RNA from cells, and use qPCR to compare expression of their candidate gene in normal cells to expression in miR-overexpressing cells. The module requires students to reproduce their results, and gives them the opportunity to make a real contribution to a cutting edge field of scientific inquiry.
Specifically, our learning objectives for this lab module are:

- Understand the use, and limitations of biological databases and prediction algorithms
- Formulate new hypotheses
- Understand the importance of controls in experimental designs
- Understand common techniques used in molecular biology and their use to address scientific questions
- Understand the importance of quantitative measurements and reproducibility in science

The module that we have developed has been run in ten different quarters of a medium enrollment (70-100 students) upper division molecular biology lab course. Two different instructors (in addition to the developers of this module) have successfully implemented this module. In our labs, the students meet for a common lecture (110 mins. each) by the instructor, and then are split into 4-5 lab sections of twenty students each. Each lab section is run by a graduate student TA, and the students work through the activities of the lab module in pairs. However, the module is easily adapted to a number of different contexts, since the technical complexity of the activities is not very high.

The module as carried out at UC Irvine spanned three 4-hour lab sessions, with one week between consecutive lab sessions. However, there are a number of stopping points in the procedure, so that with appropriate modifications, the module can be used in shorter labs (although it would then require a greater number of lab sessions). In our implementation, all the activities for a given lab session were easily completed by the students within the allotted lab time for each session.

Pre-requisite Student Knowledge

We believe that this module is ideal for intermediate to upper division students, and a basic background in molecular biology is strongly recommended. Students will need to know what RNA is, how RNA can be converted to DNA using RT-PCR, and how PCR works. Lectures during the lab course can address more advanced topics such as primer design, positive and negative controls for qPCR (quantitative PCR), and miR biology. Basic lab skills, such as dilution calculations, and pipetting will be required to successfully carry out the experiments. Basic data mining, including searching on Google and PubMed will be required to evaluate the miR target list.

Instructor Set-up

Prior to the lab sessions, the instructor will need to have transfected cells with the miR-mimic or the scrambled miR control. Following transfection, the cells will need to be collected, aliquoted and frozen down for use in the lab session. The cell culture and transfection protocols used are pretty standard, and are easily carried out, even with limited cell culture experience. To start off, one could even just use yeast cells to search for targets (in which case the transfection protocols would need to be appropriately modified).

After the first lab session (in which students will design their own primers), the instructor will need to order the student-designed primers so that the primers arrive, and are ready for use by the next lab session.

Beyond these two tasks, the load on the instructor is fairly minimal, since the procedure uses standard kits that contain all the reagents required for the lab module. At the end of Session 2 and 3, the instructor will need to send out the qPCR results to the students.

It is expected that the instructor is familiar with the following:

- Design of qPCR experiments
- Various controls used in qPCR
- Primer design
- All of the databases and software used in this module (or alternatives)

Student Outline

Session 1

Micro RNAs (miRNAs) are a class of small (20-22 nucleotide) RNA molecules that were discovered only recently (in 1993), but have since been shown to be key regulators of gene expression with roles in development, aging, cancer, and many other diseases (Banerjee and Slack 2002; Ke et al. 2003; He and Hannon 2004). It is now believed that as much as 4% of the human genome could code for miRNAs! miRNAs result from the processing of longer RNA precursors that are cleaved by specific proteins into the mature mRNAs (Griffiths-Jones 2004; Lim et al. 2003). Figure 1-1 outlines the general scheme for the generation of mature, active miRNAs from their precursors. How the production of miRNAs is controlled, and how specific miRNAs are produced in response to specific signals is an area of ongoing research.

Once produced, miRNAs can bind to complementary sequences on their target mRNAs. Thus, any mRNA (and be very careful with the use of mRNA and miRNA – that one little "i" makes a pretty big difference!) that contains a complementary sequence to a particular miRNA is a (potential) target for that miRNA (Figure 1-2).

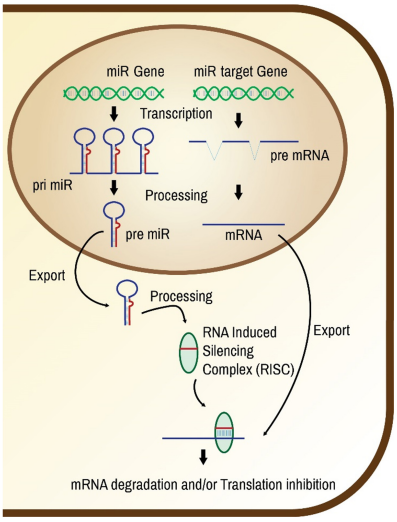


Figure 1-1. Generation of miRNAs and their effects on gene expression.

A miRNA works by recruiting a protein complex called the RISC (RNA Induced Silencing Complex) to the miRNA targets. The RISC decreases expression of the target gene by a variety of different mechanisms (Kloosterman and Plasterk 2006; Ambros 2004), the most important of which are translational repression and mRNA degradation (Figure 1-1).

It may therefore seem relatively straightforward to identify the targets of a given miRNA. All we would need to do is search the genome of an organism for sequences that are complementary to our miRNA of interest. All the genes that contain such complementary sequences would be targets of our miRNA. However, things are significantly more complicated than this (Akbari Moqadam, Pieters, and den Boer 2013). Firstly, complete complementarity is not an obligate requirement for a miRNA to target a gene. Even a partial match might be sufficient, and we do not understand the complete set of rules that govern target choice for miRNAs. Secondly, genes that contain sequences that are highly complementary (though not completely complementary) to a miRNA might not be targets for that miRNA (Figure 1-2). Thus, the identification of targets for a specific miRNA is not a trivial task.

But Why Is It Important to Identify MiRNA Targets in the First Place?

The answer lies in the importance of miRNAs to health and disease (for a listing of miRNAs associated with different diseases, see *http://www.mir2disease.org/*). Since a single miRNA can regulate multiple genes, it is important to know what the targets of a miRNA are, in order to understand its effects on the organism. For example, we can now convert skin cells into pluripotent stem cells simply by treating the skin cells with the appropriate combination of miRNAs (Anokye-Danso *et al.* 2011). Or, if a certain disease is caused by abnormal expression of a gene and we know what miRNA targets this gene, then we could inject this miRNA, causing the abnormally expressed gene to be shut off, and in theory, cure the disease (Chen, Gao, and Huang 2014).

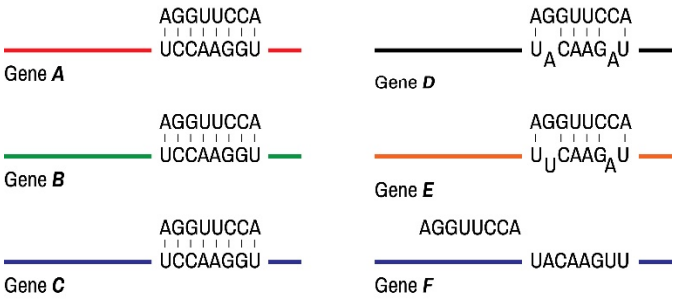


Figure 1-2. A single miRNA can have many targets. For simplicity, the miRNA is shown as a 8-mer (although miRNAs are usually 20-22 nt in length). A given miRNA can target many genes (shown here as different colored lines) as long as they contain a sequence that is complementary to the miRNA sequence. However, even partial complementarity (Genes D and E) might be sufficient for a miRNA to target a particular gene. Other genes with partial complementarity (Gene F) are not targets of the miRNA.

Alternatively, a disease could be caused by misregulation of gene expression because a particular miRNA is not being correctly expressed. In these cases, the therapeutic strategy would be to change expression of the miRNA itself, thereby correcting the imbalance in gene expression, curing the disease (Chen, Gao, and Huang 2014; Tay et al. 2014). And in fact, miRNA therapies are currently being investigated for a number of different diseases in labs all over the world (want to know more? Start here:

http://circres.ahajournals.org/content/110/3/496.full). In this lab, we will be attempting to identify the targets of a miRNA called **hsa-miR-128a-3p** which will also be referred to in this manual simply as miR-128 (as a side note on miRNA nomenclature, read this: *http://www.mirbase.org/blog/2011/04/whats-in-a-name/*). miR-128 is thought to play some role in the development of different kinds of cancers, and overexpression of miR-128 might be able to inhibit growth of tumors (Zhuang et al. 2015; Adlakha and Saini 2013). Understanding *how* miR-128 affects the development and progression of cancer, therefore, will help us develop new therapeutic strategies to deal with cancers that are affected by miR-128. The first step in this process is to figure out what genes are regulated by miR-128.

As you go through this lab exercise, remember: This module is *real, authentic* research. No one knows all the targets of miR-128, and someone in this class might be the first to discover a novel target of miR-128! How exciting is that?!!!

In order to find targets of miR-128, we will need to do the following:

1. First, pick a candidate gene that you think might be targeted by miR-128.
2. Design PCR primers specific to your candidate gene.
3. Extract total RNA from cells that are overexpressing miR-128 or a control miRNA.
4. Make cDNA from the RNA.
5. Measure the relative amount of your candidate gene's mRNA using qPCR to determine if it is a target of miR-128.

1A: Picking a Candidate Gene

The first step in this experiment will involve picking a candidate gene. You will first use ***http://www.targetscan.org/*** and ***http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi*** to identify likely targets of miR-128. These sites use different bioinformatics algorithms to predict candidates that might be regulated by miR-128. When you use these sites, remember to search specifically for hsa-miR-128. Otherwise, you might end up with irrelevant results! Play around with these websites and explore the links. What information does each link give you? How might these different bits of information be useful to you? Exploring the websites and the links is going to be important later on, so don't skip this step!

In using these resources to identify likely candidates, ponder the following questions:

- *Why do the different websites return different results?*
- *Is one site better at predicting results than the other? How might you test if one site is better than the other?*
- *If a gene is predicted to be a target by both sites, is it a stronger candidate? How might your test this as a general prediction?*

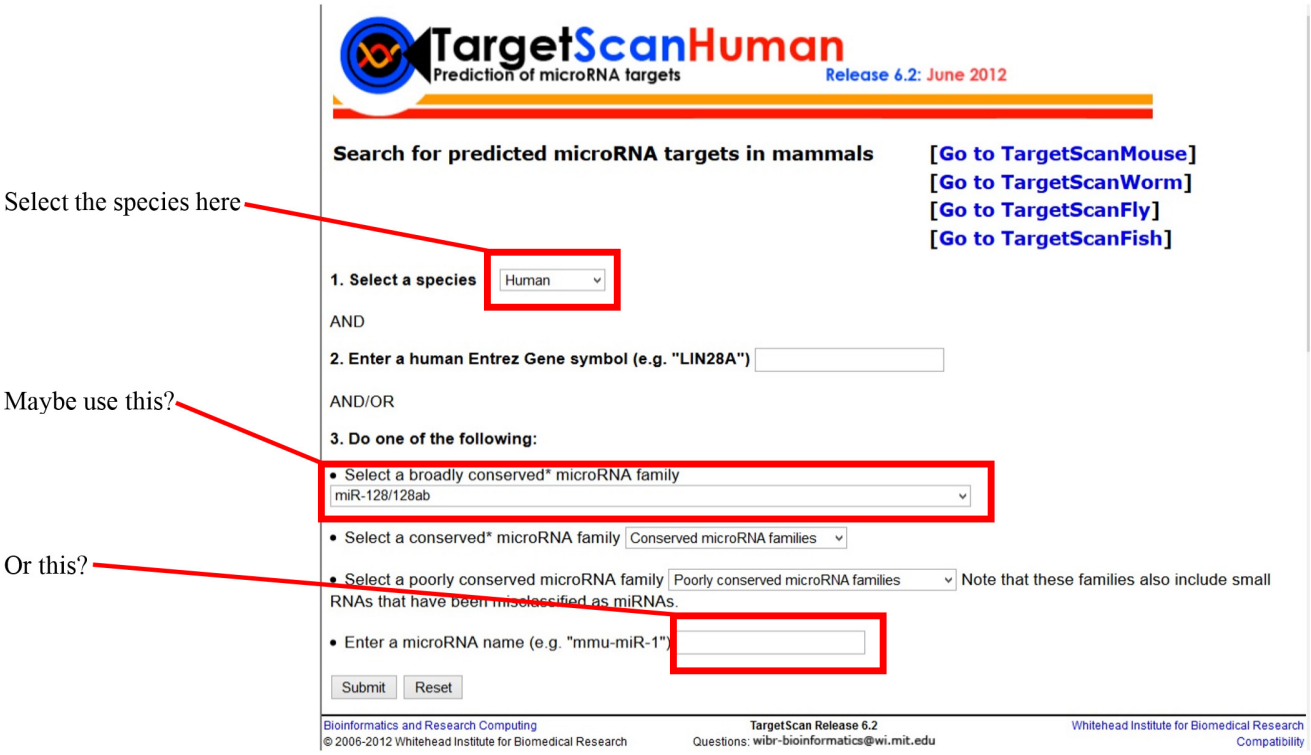


Figure 1-3. Using TargetScan to find predicted targets of miR-128.

As a researcher in the field of miRNAs, these are all questions that you would grapple with in the course of your experiments, and to which, very often, there are no clear-cut answers. So, welcome to the world of real research!

Each group will pick one candidate gene to test. Some of the things to keep in mind when you are picking your candidates are:

- *Strength of prediction (does this even matter?!)*
- *What is known about the roles of miR-128 in cells?*
- *What is known about the roles of your candidate gene in cells?*
- *Is there a plausible connection between the roles of miR-128 and your candidate gene in cells?*

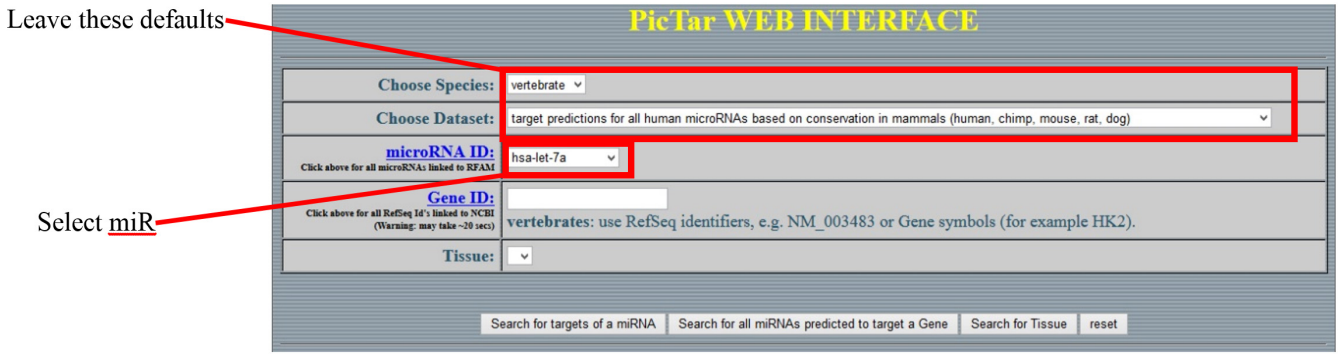


Figure 1-4. Using PicTar to find predicted targets of miR-128.

Is This Important?!

Keep in mind that the answers to these questions will be important to highlight in your lab report, when you describe why you decided to test a particular candidate gene. Some resources that will help you answer these questions are:

- *Pubmed*
- *Google Scholar*
- *Google Search*
- *Bing Search*

Once you have picked a candidate gene, go ahead and claim it in the Google Docs spreadsheet (information on this will be given to you in class/lab).

You cannot use a candidate gene that has already been claimed by another group. If all entries in the spreadsheet are not filled in, then the gene has not been claimed! So make sure that you fill in all the columns to successfully claim your candidate gene.

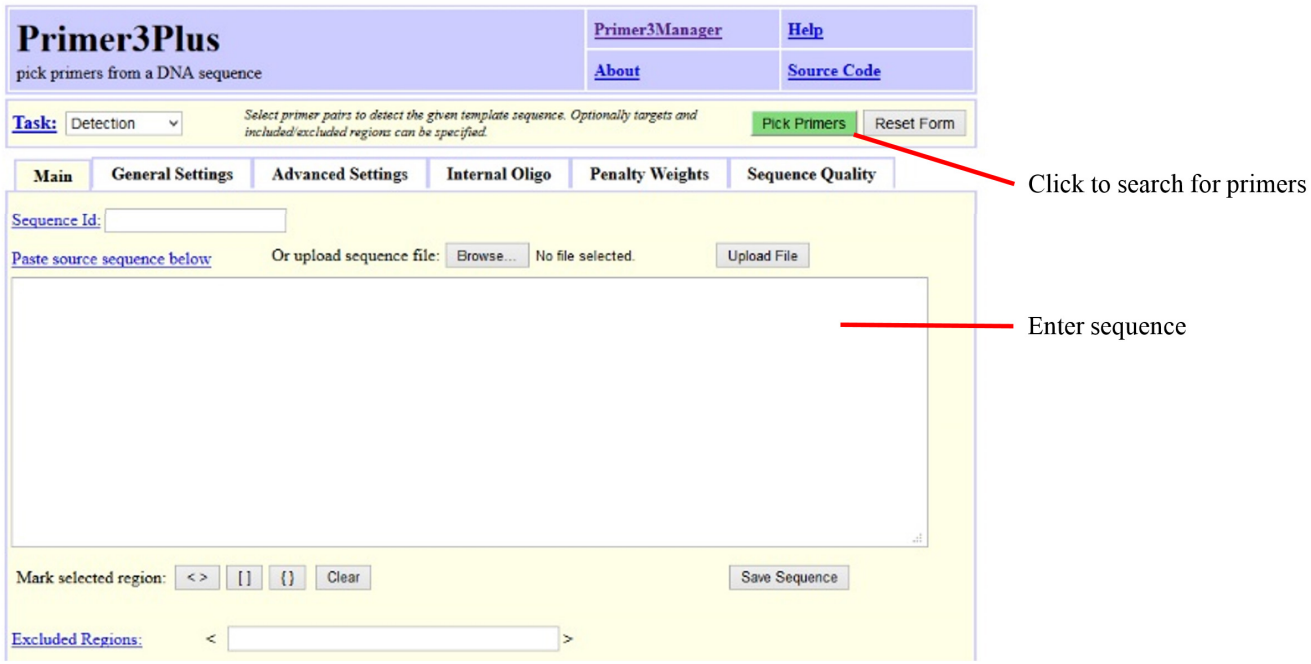


Figure 1-5. Using Primer3Plus to find primers for amplification of your candidate gene.

1B: Designing Primers To Test Your Candidate Gene

Once you have claimed your candidate gene, the next step is to design primers that will be used to test the expression of your gene of interest. The primers you design will be used to amplify your candidate gene by PCR (recall what we have learned about PCR in the previous classes). The first thing you need to design primers is the sequence of your candidate gene (will you use the genomic sequence of the gene, the mRNA sequence of the gene, or the cDNA sequence of the gene? Why?). Obtain the correct sequence you are going to use from a link on the candidate prediction website you used in part 7A (identifying your candidate gene). Check if there are alternate versions of the gene you need to take into account (alternate splicing? RNA editing?). Then, figure out a good set of primers to use. Ideally, your predicted PCR product should be around 150-250 bp in length. The parameters for good primers are (roughly in order of importance):

- *The primers are SPECIFIC to your sequence (they don't amplify anything else!)*
- *The primer pair (Forward + Reverse) shows no self-complementarity (why is self-complementarity bad?)*
- *The primer does not form step-loop structures*
- *The T_m of each primer is 55°-65°C*
- *Length is 18-30nt*
- *Usually ends in a C/G (called a CG-Clamp – why might this be good?)*
- *There are no large differences in the T_m between the forward and reverse primer*
- *There are no nucleotide "runs" (stretches of more than 3 of the same nucleotide) in the primers*

It is certainly possible to manually stare at the candidate sequence and come up with primers that satisfy most of these criteria. However, it is much easier and usually more accurate to use a computer to initially identify the best candidates and then manually pick the best ones from this subset of primers. There are many, many, many different algorithms and software that will pick primers for you, and most of them are very good. However, for this lab, we will use ***www.bioinformatics.nl/primer3plus/*** to design primers (Why you ask? –it's simple to use, it works pretty well, and it's free!). Closely examine the output and pick a set of primers to use.

Double check the primers using:

- ***http://www.basic.northwestern.edu/biotools/OligoCalc.html***
  Enter each primer, and use this program to calculate the primer parameters, and check the predicted self-complementarity (why might some of the parameters not match the Primer3 output?)

- ***http://www.ncbi.nlm.nih.gov/tools/primer-blast/***
  Then, use the PrimerBLAST tool (BLAST is a way of checking for sequence similarity) to check if your primer pair is specific (i.e., they don't show amplification of other products).

Using all these tools, you should have ensured that the primers satisfy the criteria listed above. *(How will you decide between pairs that appear very similar to each other?)*

Once you've picked a set of primers, enter them in the **Google Docs spreadsheet** (information on this will be provided in class/lab).

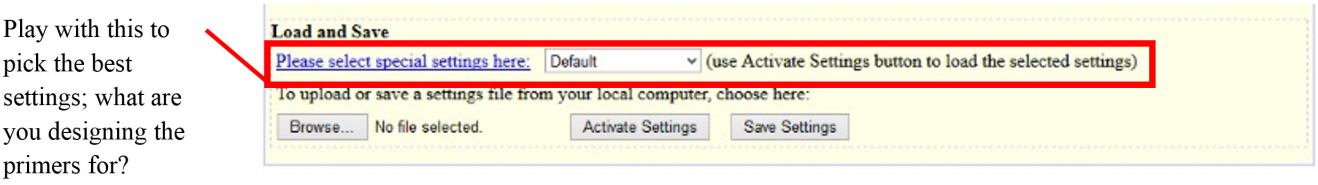


Figure 1-6. Optimizing settings for primer design in Primer3Plus using the "General Settings" tab.

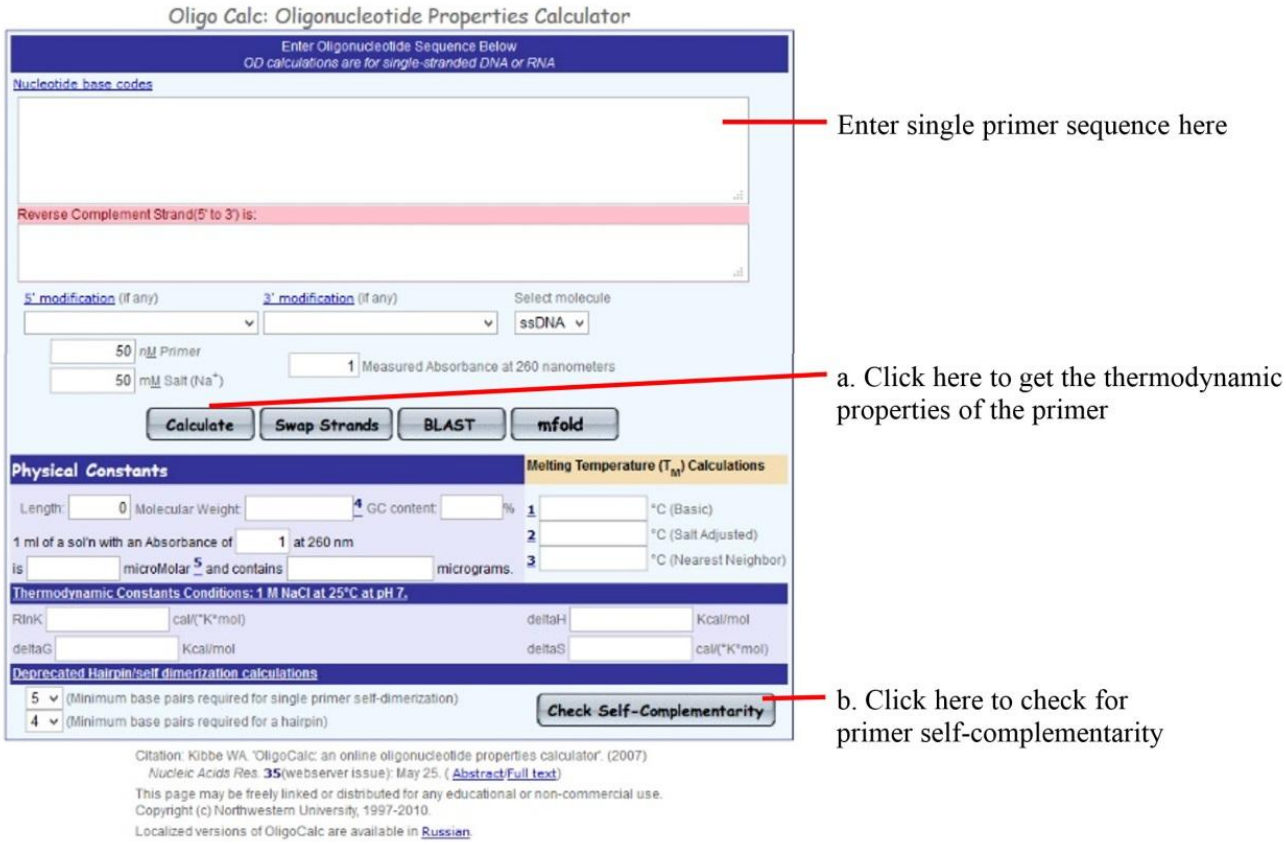


Figure 1-7. Oligonucleotides properties calculator.

Make sure that all the columns of information are filled out correctly. Most importantly, make sure you have entered the correct primer sequence (probably best to use copy-and-paste from the primer prediction website), and that it is in the correct orientation. We will order the primers for you, and they will be ready to use in the next lab. When you get your primers, you will need to dissolve them in **water** to get a **final concentration of 100µM**.

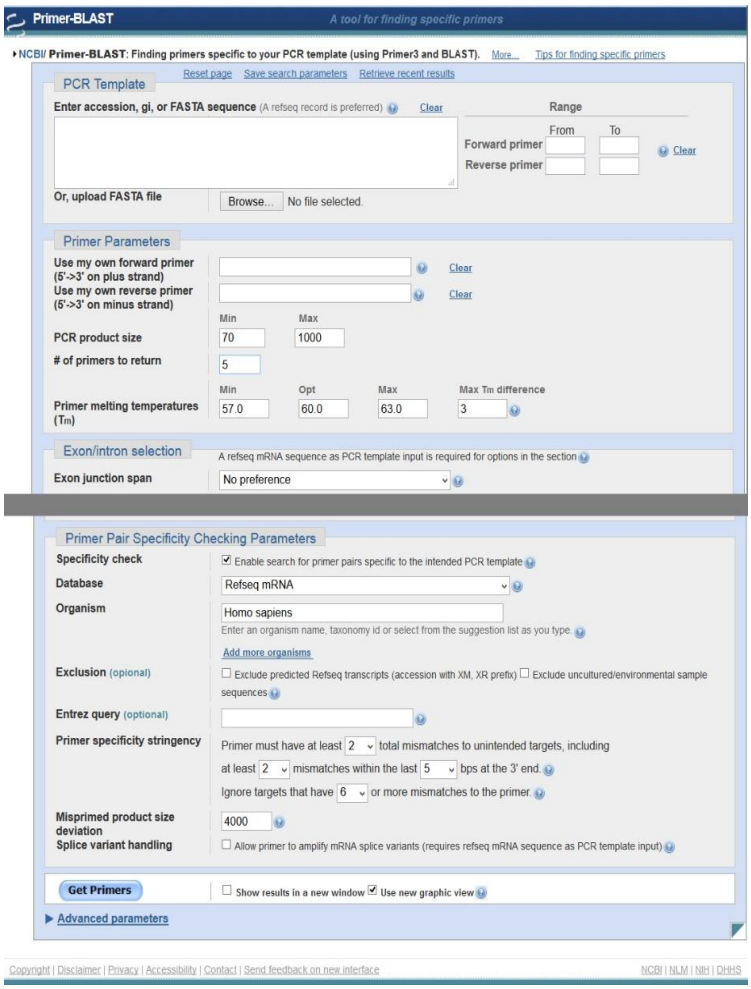


Figure 1-8. Checking for mispriming using PrimerBLAST.

Student Outline: Session 2

2A: Isolation of Total RNA from Cells

Now that you have chosen your candidate gene and designed primers specific for it, the next step is to isolate RNA from HeLa cells that have already been transfected with miR mimics for either miR-128 or a control "scramble" sequence. Mimics are short double-stranded RNAs that are designed to mimic an endogenous miRNA, which causes an up-regulation of the miRNA's activity. The scramble mimic is designed specifically to not mimic any known endogenous miRNA. In order to generate your samples, HeLa cells were transfected using DharmaFECT1 Transfection Reagent from Thermo Scientific. This transfection process (there are many others including chemical-based, electroporation, optical with lasers, magnet assisted, and more) uses liposomes to introduce the miR-mimic and scramble RNA into the cells by taking advantage of the negative charge of the RNA. (*Why might one transfection process be better than another?)* The lipids in the transfection reagent form a vesicle around the RNA, which then fuses to the phospholipid bilayer and releases the genetic material into the cells. At 48 hours post-transfection the cells are passaged (cells are allowed to grow in fresh media), and then aliquoted at $5x10^5$ cells per microfuge tube and pelleted by centrifugation. The supernatant was discarded and the pellet was frozen at -80ºC.

On the day of the lab the cells will be lysed and the RNA will be isolated and purified using the GeneJET RNA Purification Kit from Thermo Scientific. (*Why do we need to purify the RNA?)* The lysis buffer used in this kit contains guanidine thiocyanate, which is a chaotropic salt that disrupts and denatures RNases. When mixed with ethanol, this salt causes the RNA to bind to the silica membrane on the column used during washing. Impurities can then be removed using wash buffers and centrifugation. *What causes the RNA to remain on the column during the washing steps?* The ethanol and high ionic strength of the wash buffers!

In the final step, the RNA is eluted using nuclease-free water (low ionic strength) and collected in a clean microcentrifuge tube. Once we have purified the RNA we will use it to make cDNA.

To purify your RNA from your cell pellets using the GeneJET RNA Purification Kit from Thermo Scientific you will need six ingredients (*Why do you need the miR-Scramble cells? How are they a "control"?*):

1. Frozen Cell Pellets in microcentrifuge tubes (1 for the miR-scramble and 1 for the miR-128 mimic)
2. Lysis buffer supplemented with β-mercaptoethanol (*why do we add β-mercaptoethanol?*)
3. Ethanol (*what happens to the structure of RNA when ethanol is added?*)
4. Wash Buffer 1 supplemented with 96-100% ethanol
5. Wash Buffer 2 supplemented with 96-100% ethanol
6. Water, nuclease-free (*why does the water have to be nuclease-free?*)

Protocol – RNA Isolation

1. Resuspend cell pellet in **600µL** of **Lysis Buffer** by pipetting. Vortex for 10 seconds.
2. Centrifuge the tubes for **5 min** at **12000xg** and transfer the supernatant to a new sterile RNase-free microcentrifuge tube.
3. Add **360µL of (96-100%) ethanol** to each tube and mix by gently pipetting.
4. Transfer **700 µL** of each lysate to a Purification Column **inserted in a collection tube**.
5. Centrifuge columns for **1 min** at **12000xg**. Discard the flow-through in the collection tubes and place the purification columns back into the collection tubes. Repeat steps 4 and 5 until all of the lysate has been purified (at this point our RNA is attached to the silica membrane in the purification column).
6. Add **700 µL of Wash Buffer 1** to columns.
7. Centrifuge columns for **1 minute** at **12000xg**. Discard the flow-through in the collection tubes and place the Purification Columns back into the collection tubes.
8. Add **600 µL of Wash Buffer 2** to columns.
9. Centrifuge columns for **1 minute** at **12000xg**. Discard the flow-through in the collection tubes and place the Purification Columns back into the collection tubes.
10. Add **250 µL of Wash Buffer 2** to columns.
11. Centrifuge columns for **2 minutes** at **12000xg**. Discard the flow-through in the collection tubes and place the Purification Columns back into the collection tubes.
12. Re-centrifuge the columns for **1 minute** at **12000xg**. (*Why do we do this step?*)
13. Place the Purification Column into a sterile **1.5 mL** RNase-free microcentrifuge tube. You can now discard the collection tube, because our RNA is ready to be eluted from the membrane on the column.
14. Add **50 µL of nuclease-free water** to the center of the silica membrane on both purification columns. This is the elution step, so the water must be in contact with the membrane where the RNA is. While adding the water, make sure that you **DO NOT** touch the membrane with your pipette tip.
15. Incubate at **room temperature** for **3 min**.
16. Centrifuge columns for **1 minute** at **12000xg** to elute the RNA into the sterile 1.5 mL RNase-free microcentrifuge tube. Retain the purification column in case an additional elution step is needed (it can be discarded at the end of class).
17. Split the RNA into two aliquots, and immediately freeze one aliquot at -20°C.
18. Congratulations! You now have purified RNA. Proceed to performing reverse transcription to create cDNA.

2B: Making cDNA from Total RNA

Once the RNA is isolated, it should represent a snapshot of gene expression (mature mRNA) in cells treated with either miR-scramble control or miR-128. Our overall goal is to use the primers designed in lab 1 to detect relative expression of your candidate gene in miR-128 treated cells compared to miR-scramble control treated cells. In this section you will take the isolated RNA and convert it into complementary DNA (cDNA). *Why can't we just use the isolated RNA and "count" the copies of our candidate gene?* There are two reasons. First, single stranded RNA is highly unstable meaning that it is sensitive to degradation by RNases (enzymes that degrade single stranded RNA). Secondly, the method used to detect relative expression of your candidate gene is based on a fluorescent dye inserting itself (intercalating) between double stranded DNA.

Beginning with single stranded RNA, we will take advantage of reverse transcriptase, an enzyme isolated from a virus that uses an RNA template to generate a complementary DNA strand. All DNA polymerases are primer dependent and so we use both oligo $(dT)_{18}$ which is a short sequence of 18 deoxy-thymine nucleotides (*why would we use this and where on the mRNA would it bind to?*) as well as random hexamers which are a mixture of single-stranded random hexanucleotides (*where*

on the mRNA would these bind to? Why do we need a mixture of hexamers?). In order to synthesize cDNA, the enzyme needs the raw material or building blocks for the new cDNA strand and so we also add a mixture of the fours dNTPs.

RNases exist everywhere and are a common defense in the human body to destroy free-floating single stranded RNA that is generally viral in origin. One precaution when working with RNA, and when performing the cDNA synthesis is to **not talk, breathe, sneeze, or cough over your tubes**, otherwise RNases could make it into your tube and destroy your RNA!

In order to generate cDNA you will use the Maxima First Strand cDNA Synthesis Kit from Thermo Scientific. To setup the reaction you will need four ingredients:

1. 5X reaction buffer. This contains your reaction buffer (salts), dNTPs, oligo dT and random hexamer primers.
2. Maxima enzyme mix. This contains the reverse transcriptase as well as RNase inhibitor.
3. Water, nuclease-free (why does the water have to be nuclease free?)
4. Template RNA. Single stranded RNA isolated in 2A.

Note: It is very important to keep anything with the Maxima enzyme mix on ice until the reaction is ready to place into the thermocycler.

Protocol – cDNA synthesis

1. Add the reagents shown in the table into sterile, RNase-free PCR tubes on ice in the indicated order (from left to right). *Why do we include the tubes without the reverse transcriptase enzyme mix added (-RT)*?

Tube	Sample template	5X Rxn buffer	Maxima enzyme mix	Template RNA	Water, nuclease-free	Total volume
1	Ctl+RT	4 µl	2 µl	1 µl	13 µl	20 µl
2	Ctl-RT	4 µl	0 µl	1 µl	15 µl	20 µl
3	miR+RT	4 µl	2 µl	1 µl	13 µl	20 µl
4	miR-RT	4 µl	0 µl	1 µl	15 µl	20 µl
NOTE: Ctl = cells transfected with the miR-Scramble						

2. Cap tubes and flick gently to mix. Briefly centrifuge to collect reaction to the bottom.
3. Place tubes in a thermocycler using the following protocol: **10 min** at **25˚C**. **15 min** at **50˚C**. **5 min** at **85˚C**. Hold at **4°C**. Now you have cDNA! Proceed to the qPCR.

2C: Quantifying gene expression using qPCR

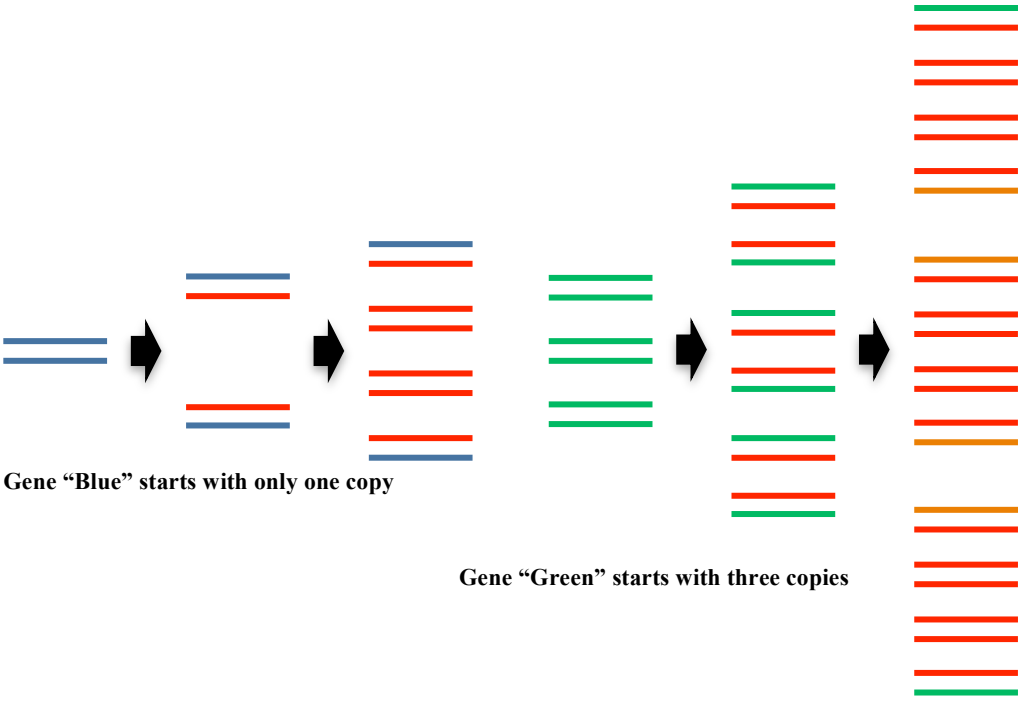


Figure 2-1. The initial number of PCR products is very sensitive to the amount of starting template. The "Blue" gene has only one cDNA molecule, and at the end of 3 cycles of qPCR results in 4 ds products. However, the "Green" gene starts off with 3 copies, and at the end of 3 cycles of qPCR results in 12 ds products. Thus, measuring the amount of product in the early rounds of PCR gives us a very accurate and sensitive estimate of the starting template amount.

You have finally synthesized your cDNA from the RNA isolation step - but now what? Now it's time to start the *actual* experiment of quantifying the candidate gene expression in the lysed cells treated with either miR-scramble control or miR-128. Compared to RNA, the cDNA synthesized is quite stable, as it exists as tightly paired double stranded structure. Though relatively stable at 4°C, ***properly*** stored cDNA has been found to be useable even after 16 years!

You will quantify gene expression using the primers you designed in Lab 1 to specifically amplify your candidate gene by PCR. You will employ a variation of PCR called **quantitative PCR** (qPCR) to estimate the relative expression level of your candidate gene. qPCR couples DNA amplification with detection in real time (*will you need to run a gel at the end of qPCR?*) to give a very accurate estimate of the amount of target sequence present in the PCR reaction. Remember that in the initial stages, the rate limiting step in PCR is the amount of template DNA (*Why?*), so the amount of product formed will be directly proportional to the amount of template DNA present. Thus, by measuring the amount of product in the early cycles of PCR, we can get a very accurate and sensitive estimate of the amount of template present in the reaction (Figure 2-1). If your candidate gene was a target of miR-128, you would then expect to see a difference in the amount of the starting cDNA template (and hence, the original mRNA) between the miR-128-treated cells, and the controls (*what difference do you expect to see*?).

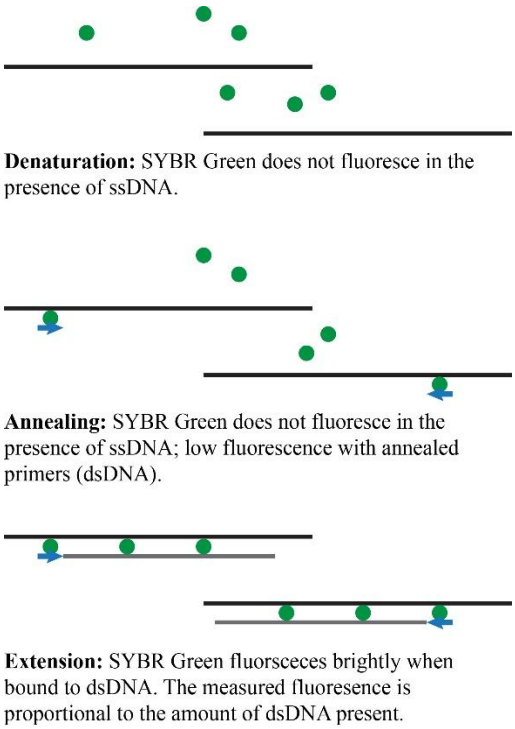


Figure 2-2. Principle for SybrGreen qPCR.

We will be using fluorescent dyes in our qPCR to label the PCR products produced and quantify the relative amounts of amplified target gene DNA. Specifically, we will use "SYBR Green", which binds to *all* double-stranded DNA and detection of your target gene is monitored at each PCR cycle by increases in fluorescence (see Figure 2-2).

Before one starts the qPCR with their cDNA, the issue of normalization needs to be addressed. Imagine a scenario where you find that your miR-treated cells have 50% of you candidate mRNA when compared to controls. However, you also started off with 50% less ***total*** RNA in your miR-treated cells when compared to controls. Now, can you make any conclusions about your candidate gene? Therefore, when doing qPCR, one needs to be assured that the total amount of input RNA is equal across all samples (or that we can normalize our final values based on the amount of total starting RNA). This doesn't mean that we are looking to make sure that the starting RNA of your ***actual target gene*** is the same (then there would be no difference in gene expression!), rather we are looking to make sure the total cellular amounts of RNA from your cells is comparably equal. To do this, a housekeeping gene control is introduced across all the samples to be run, and we pick a gene that is known to NOT be targeted by our miR (*why is it important that the gene not be target?*). A housekeeping gene is defined as any reference gene found in your cell type that will not fluctuate in expression levels when subject to the same treatments or conditions that one has performed on the cells. For our experiment, we will be using Beta-2 microglobulin, a MHC class I molecule present in all nucleated cells.

Further, we will need to include a positive control, to make sure that our experimental conditions are working. What do you think is a good positive control for our experiment?

To set up the qPCR, we will use the Thermo Scientific Luminaris Color Hi ROX qPCR Master Mix from Thermo Scientific. Here is what is needed to setup the reaction:

1. 2X qPCR master mix (contains your Hot Start Taq DNA polymerase, dNTPs, and optimized PCR buffer)
2. Water, nuclease-free
3. 40X Sample Buffer
4. Template cDNA obtained from 8B
5. Forward primers
6. Reverse primers

Note: Keep everything on ice until the reaction is ready to place into the thermocycler.

Protocol - qPCR

1. **NOTE**: If you have not filled in the information in the **spreadsheet correctly**, we cannot track your samples, and you will not get any results back.
2. Prepare your template DNA for each sample you will be using, using the table below. This diluted, yellow sample is referred to as the "**Final sample**."

Tube	Sample	Water	DNA	40X Yellow Sample Buffer
1	+RT (Ctl)	16µL	4µL	5µL
2	-RT (Ctl)	16µL	4µL	5µL
3	+RT (miR)	16µL	4µL	5µL
4	-RT (miR)	16µL	4µL	5µL

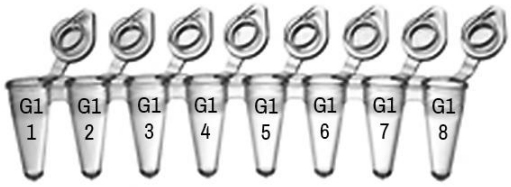
3. Prepare your Primer Mix for each set of primers, so that a Primer Mix contains both forward and reverse primer at a concentration of **10µM** each. You should have **three** different Primer Mixes – one for the Endogenous control, one for the Positive control and one for the candidate gene.
4. Add the reagents shown in the table into a sterile, 8-strip PCR tube on ice in the indicated order (from left to right). *Keep track of what sample is in which tube by correlating the tube number with the sample in it*.

Tube	Template	Primer Mix (1.2µL of F+R mix)	2X Master mix	Final Sample	Water, nuclease-free
1	+RT (Ctl)	END control[1]	10 µl	2.5 µL	6.3 µL
2	+RT (Ctl)	POS control[2]	10 µl	2.5 µL	6.3 µL
3	+RT (Ctl)	Candidate gene	10 µl	2.5 µL	6.3 µL
4	-RT (Ctl)	END control [1]	10 µl	2.5 µL	6.3 µL
5	+RT (miR)	END control [1]	10 µl	2.5 µL	6.3 µL
6	+RT (miR)	POS control[2]	10 µl	2.5 µL	6.3 µL
7	+RT (miR)	Candidate gene	10 µl	2.5 µL	6.3 µL
8	-RT (miR)	END control [1]	10 µl	2.5 µL	6.3 µL

[1]END control: Endogenous control, *Beta-2-microglobulin* (B2M); [2]POS control: Positive control, *Bmi1*

IMPORTANT: Remember to label your tubes with your unique Group number or we will not be able to identify your samples (See example image below)!!!
IMPORTANT: Do NOT label the caps of the tubes or the qPCR machine CANNOT read the fluorescence in your samples!
REALLY IMPORTANT: Do NOT label the caps of the tubes!

5. Cap tubes and flick gently to mix. Briefly centrifuge to collect reaction to the bottom.



6. Place tubes in the qPCR machine using the following protocol: 10 min at 95˚C15 sec at **95˚C, 30 sec at 60˚C, and 30 sec at 70˚C - for 40** cycles. Hold at **4°C**.
7. Your cDNA has been qPCR'd! Your data will be given to you for analysis!

For the next lab, you will repeat the qPCR.

Using your qPCR results ($\Delta\Delta C_T$ method; Adapted from http://blog.mcbryan.co.uk/2013/06/qpcr-normalisation.html)

You must calculate the ***Delta Delta CT*** for your target gene and the Positive control using the following information

$$\Delta\Delta CT = \Delta CT_{(control)} - \Delta CT_{(miR)}$$

Where:

$$\Delta CT_{(control)} = CT_{(target:control)} - CT_{(endogenous\ control:control)}$$
$$\Delta CT_{(miR)} = CT_{(target:miR)} - CT_{(endogenous\ control:miR)}$$

You can then calculate the **ratio of expression as** $2^{-\Delta\Delta CT}$

Remember that higher expression means that your threshold cycle will be lower! Also pay attention to your –RT readings. What does it mean if they are high? What does it mean if they are low?

Worked Example

miR	
Sample	CT
Endogenous	14.7492
Positive	28.1164
Target	28.8212
Endogenous	Undetermined
Positive	Undetermined
Target	Undetermined

(Note: Undetermined = no amplification)

Control	
Sample	CT
Endogenous	15.5057
Positive	26.7152
Target	25.4664
Endogenous	Undetermined
Positive	Undetermined
Target	Undetermined

miR	Control
$\Delta CT_{(target)} = 28.8212 - 14.7492 = 14.072$	$\Delta CT_{(target)} = 25.4664 - 15.5057 = 9.9607$
$\Delta CT_{(positive)} = 28.1164 - 14.7492 = 13.3672$	$\Delta CT_{(positivel)} = 26.7152 - 15.5057 = 11.2095$

$\Delta\Delta CT_{(target)} = 9.9607 - 14.072 = -4.1113$
Ratio of expression = $2^{-4.1113} = 0.056$

$\Delta\Delta CT_{(Positive)} = 11.2095 - 13.3672 = -2.1577$
Ratio of expression = $2^{-2.1577} = 0.224$
Therefore Target IS decreased by miR overexpression (positive control worked, shows decreased expression).

Materials

Note: Students will work in pairs, so the equipment and supplies listed are for 24 students, or 12 pairs of students.

Preparation for Lab – Transfection of Cells:

1. HeLa (or other easily transfectable human cell line)
2. HeLa growth medium (or other growth medium appropriate for the chosen cell line)
3. Scrambled miR control and miR-128 mimic
4. DharmaFect (or other appropriate transfection reagent for transfecting cells)
5. Tissue culture-treated flasks for growing cells
6. Pipettes
7. Pipettor
8. Cell culture hood
9. Sterile PBS

Lab Sessions 1 and 2 (Lab session 3 is a repeat of 2)

1. Each pair should have at least one computer for the dry lab part of the lab (Session 1).
2. 12 tubes of frozen cells transfected with control miR.
3. 12 tubes of frozen cells transfected with miR-128.
4. GeneJet RNA purification kit for 24 RNA preps.
5. Maxima First Strand cDNA synthesis kit for 48 reactions.
6. Luminaris Color HiROX qPCR Master Mix for 96 reactions.
7. Endogenous control PCR primers for amplification of *B2M* (25nmolar synthesis is sufficient)
8. Positive control PCR primers for amplification of *Bmi1* (25nmolar synthesis is sufficient)
9. Target gene PCR primers (designed by students; 25nmolar synthesis)
10. Eppendorf tubes
11. Sterile water
12. Micropipettors
13. Pipette tips
14. qPCR tubes and lids
15. PCR tubes
16. Thermocycler
17. qPCR-capable thermocycler

Notes for the Instructor

An important part of the lab is to prepare the transfected cells prior to the labs. Instructors will need to do the cell transfections with either scrambled control miR or miR-128 mimic. Cells can be transfected in a single large batch as needed, and then aliquotted into individual tubes. The individual tubes should be frozen down for use by students. It is also recommended that the Instructor test each batch of transfected cells using the Positive control, to ensure that the cells have been successfully transfected.

It should also be noted that the protocols listed for the lab sessions are based on the *specific* kits and reagents that we have used (and which are listed here). However, if you choose to use different kits/reagents, please modify the student handout appropriately. We chose these kits based on our limited testing of the available resources. They work well, but they are certainly *not* the only option. Feel free to use a cheaper/quicker/more available set of reagents as you see fit.

A flow chart for the module is shown below, and provides an overview of the lab activities:

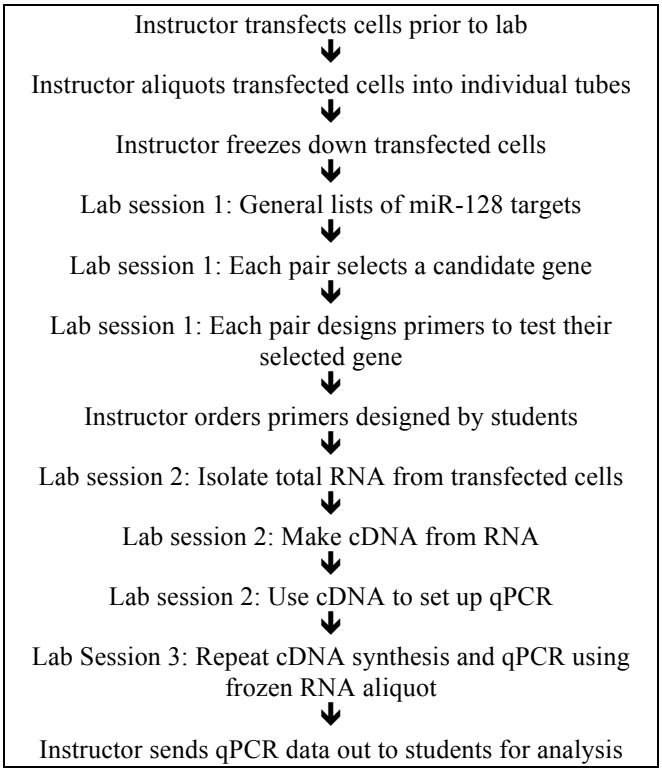


Figure 3-1. Flowchart of lab sessions and activities.

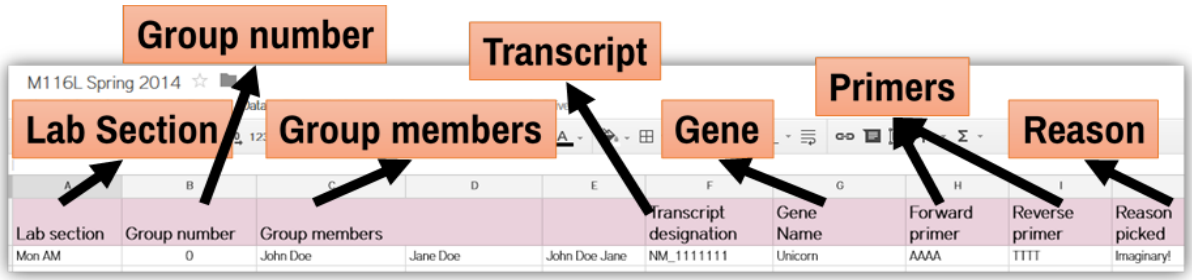


Figure 3-2. Main components of the Google spreadsheet to collect student information.

Instructors should also familiarize themselves with the various online tools used in the dry lab session, and set up a Google Docs spreadsheet as shown in Figure 3-2.

Prior to lab, instructors will need to go over (with students) the basics of miRs, miR target prediction, criteria for selection of interesting miR candidate genes, PCR, PCR primer design, RT-PCR and qPCR.

Once students have designed the primers for their selected candidates, the instructor will need to order the primers (using the information in the GoogleSheet) so that they arrive in time for the next lab session. We use IDT for the primer orders, and with standard shipping they always arrive by the time students need to use the primers.

Sample Results

The spreadsheet shows the sample results from one of our lab sections (data taken from Winter 2014). The final fold expression results are shown, for each of the two repeats for the Positive control and the target gene. Based on this, for example, we can say that Group 8's candidate is **very** likely a miR-128 candidate. We can then refer back to the Google Spreadsheet to figure out what the target was.

Fold Expression

	+ve Control			Target gene		
	Repeat 1	Repeat 2	Average	Repeat 1	Repeat 2	Average
Group 1	0.23442	0.61089	**0.42266**	0.18747	1.14654	**0.66700**
Group 2	0.26673	ERROR	**0.26673**	0.55772	ERROR	**0.55772**
Group 3	0.25892	ERROR	**0.25892**	ERROR	0.14320	**0.14320**
Group 4	0.19246	ERROR	**0.19246**	0.46819	ERROR	**0.46819**
Group 5	0.35176	0.44606	**0.39891**	0.26471	1.69839	**0.98155**
Group 6	0.44263	0.68528	**0.56395**	0.70381	ERROR	**0.70381**
Group 7	ERROR	0.55809	**0.55809**	0.94180	1.24950	**1.09565**
Group 8	0.32963	0.31686	**0.32325**	0.21094	0.48858	**0.34976**

Figure 4-1. Table showing example results from one lab section

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