

Shhhhh... Keep it down! Designing, Making, and Testing shRNA

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The following report is a modified description of an upper-level undergraduate lab course involving RNA interference (RNAi). RNAi is an umbrella term that includes any methodology that works to interfere with the expression of a target RNA sequence and is currently a major tool used by scientists around the world to understand gene function. A cost-effective PCR-based method for producing a popular form of RNAi, namely short hairpin RNA (shRNA), was adapted from the literature for the purposes of creating a mini project that undergraduate students with basic bench research skills would complete over a 12-week semester course. For the purposes of this communication and recognizing the varied needs of the readership, the author outlines different scenarios in which shRNA against any gene transcript can be created and tested as either a short independent lab module or as a core project for an entire lab course.

Keywords: shRNA, RNAi, transfection, knock down, undergraduate, lab

Link to Supplemental Materials: <http://www.ableweb.org/volumes/vol-38/oran/supplement.htm>

Introduction

Since its discovery near the turn of the 21st century, RNA interference (RNAi) has become a popular method for scientists wishing to understand the biological function of a particular gene in the context of the cell or complete organism (for review, see Wilson & Doudna, 2013). RNAi was co-discovered by two American scientists, Andrew Fire and Craig Mello¹, who published in the late 90's their description of a natural mechanism in cells that 'interfered' with gene expression through the use of double-stranded (ds)RNA molecules as a mediator of interference. Thanks to their discovery, researchers now had a means to investigate and elucidate the function of any gene. By adding into a cell artificially-designed interfering molecules of dsRNA with specific complementarity to naturally-occurring cellular mRNA transcripts, scientists could exploit the cell's own RNAi machinery to 'silence' (or 'knock down') the targeted transcripts at will. In so doing, scientists could elaborate the role of each targeted

transcript on the phenotype of the cell and thus gain insight into their cellular functions. RNAi has been so impactful in both theory and practice that today most graduates with a postsecondary education in the biological sciences have at least heard of RNAi and its different forms, including short hairpin (sh)RNA, a popular form of dsRNA that can interfere with expression (Paddison et al., 2002). Unfortunately, at least at my institution, this knowledge did not extend beyond the lecture hall; short of working in a research lab that used RNAi strategies, the undergraduates' exposure to RNAi was limited to passive transfer from expert-to-learner via lecture or course-assigned readings.

Since RNAi is a mainstay in basic biology research as well as a core concept in cell and molecular biology education, it only made sense to provide students another authentic research experience that was pedagogically-relevant to their learning but also easy and cost-effective to implement. In 2013, a shRNA-based project was piloted at the University of Ottawa as an undergraduate lab course in which

¹ They were co-winners for the Nobel Prize in Physiology or Medicine in 2006 for their findings.

senior-level students would design, make, and test different shRNA-expressing constructs during a 12-week semester. These constructs would be altered the following school years to target other genes in part to minimize any opportunities for cheating (e.g. students benefiting from the work of the previous cohort) but also to prevent obsolescence of the teaching material.

In addition to its scholastic benefits, the course was designed to be *plug-and-play*, meaning that although the overall project, namely the shRNA and its target gene, would vary after each repeat of the course, the main process including the design, creation, and testing protocols would remain unchanged with the possible exception of the final functional assay(s) used to measure the knock down effect². This structure provided the instructor and the technical staff a measure of stability and predictability that simplified the organization and management of each lab session. Moreover, thanks to the consistency in overall course design and structure, the lab has become reasonably cost effective as any initial investment in equipment has been spread over the various iterations through the years. This is especially important when considering recent budgetary restrictions and cost increases for supplying reagents to three separate lab classes of up to 32 students per class per school year.

The main objective of this report is to share with the reader a tried-and-true strategy at designing, making, and testing shRNA as part of an undergraduate lab experience. Of the three elements listed, this report will mainly focus on the design portion, describing the steps involved in the planning of a customizable shRNA-expressing DNA construct. This portion is arguably the easiest to adapt and adopt in a teaching laboratory environment, being the most affordable and the least technically-demanding. In so doing, any student, regardless of year of study, can learn how to design their own shRNA and, at the same time, gain hands-on experience with the procedure. This bioinformatics portion of the lab can be completed in as little as 3 to 6 hours and will only require internet-connectable devices to access free internet services (links provided). That being said, the lab can be extended to include a practical component, allowing students the opportunity to synthesize their

designs using a traditional PCR-based method. In this expanded version of the course, two options are provided; option one is the least expensive and time consuming while option two (leaping off of option one) involves more steps and costs but offers some important advantages. The final aspect of the course, namely testing of the constructs, arguably the most costly and technically-demanding element, is left to the end of the report and is only possible if the necessary resources and equipment are available and to scale for the size of the class. In the end, regardless of which element or option is selected, the reader can gain some valuable pointers as to how to introduce an RNAi component in their lab curriculum. The report concludes by sharing some of the pertinent details (e.g. timeline, costs, and equipment) as well as insights to reduce costs without necessarily compromising the learning potential from working with shRNA.

² If the target was a fluorescent gene, a fluorescence-based assay would be used to measure the suppressive effect of the shRNA. If it was a kinase, a phosphorylation assay would be used to assess function.

Student Outline

Designing, Making, and Testing shRNA, Parts I to VI

OPTION 1: Part I – Bioinformatics - Designing RNAi 101

Goal: To learn how to design RNAi-expressing constructs in order to silence or “knock-down” gene expression

RNA interference (RNAi) is a natural mechanism of gene regulation first discovered back in the early 90s with petunias but later shown to play a role in animals including nematodes, insects, and mammals (as reviewed in Sen and Blau, 2006, and Scherer and Rossi, 2004). In short, the RNAi works in a sequence-specific manner to suppress mRNA translation and thereby silence gene expression (see below). Our understanding of the mechanisms by which this ‘gene silencing’ occurs has expanded to the point where labs around the world now use RNAi-based strategies to understand gene function in different plant and animal species. The ramification of this fundamental process in both the animal and plant kingdoms is not only limited to basic research; clinical scientists now have the ability to design therapeutic RNAi agents to treat various genetic disorders or create transgenic organisms. It should be no surprise, therefore, that in 2006, Drs. Andrew Fire and Craig Mello, credited for discovering RNAi, were jointly awarded the Nobel Prize in Physiology and Medicine. Today, it is hard not to find a seminal scientific publication in the medical field that does not use RNAi; a PubMed search using the keyword “RNAi” gave 18449 matches at the time of this manual preparation in 2015. In fact, the widespread use of RNAi in labs around the world was the prime motivation behind the design of this RNAi project.

As mentioned above, RNAi works in a sequence-specific manner to target and suppress mRNA translation. It appears to begin with the production of double-stranded RNA (dsRNA) that is encoded by non-protein coding genes; these dsRNA molecules are made up of a series of repeated sequences that are not only complementary to themselves but also to sequences found in certain protein-encoding mRNAs of the cell (reviewed in Sen and Blau, 2006). Once transcribed, the dsRNA that is formed is recognized by a complex of proteins which triggers a RNase-based pathway that results in dsRNA cleavage into small interfering RNA (siRNA) duplexes. These 21-25 nucleotide long double-stranded siRNAs are then unwound and the negative (antisense) strand integrates into a multiprotein complex called the RNA-induced silencing complex (RISC) (as reviewed in Agrawal et al., 2003). This antisense (-) siRNA serves as the targeting agent for the RISC, hybridizing to a complementary sequence found in the sense (+) mRNA strand. Upon binding to the mRNA, RISC mediates endonucleolytic cleavage of the mRNA, thereby destroying it (hence silencing the expression of that gene). The RISC-siRNA complex can then detach and re-associate with other complementary copies of mRNA to repeat the degradation. Depending on the extent of degradation, the level of suppression can be partial (gene knock-down) or complete (gene knock-out).

Please note that this is but ONE model to explain siRNA-based gene silencing. There are other models to explain gene silencing that you are encouraged to discover on your own, beginning with an online literature search (for example).

Since in this course we will be using a mammalian cell line, we will concern ourselves with a methodology that will allow us to suppress a gene in this context. The remaining part of this section will devote itself to the design of RNAi.

The science behind the design of RNAi for a particular gene is a topic of great interest for scientists interested in silencing a particular gene. It is important to note that not all sites along a mRNA transcript are sensitive to gene silencing by RNAi; only certain sequences appear to be targeted by siRNA (as reviewed in Castanotto et al., 2002). Not surprisingly, there has been a strong push for bioinformaticians to develop useful models to predict the locations of siRNA target sites. We will be using some of these tools to predict candidate sites. In the end, the only way to determine if a site is truly susceptible to RNAi is by experimentation.

Here are some websites available for determining possible siRNA target sites (as of the time of this publication):³

- *Integrated DNA Technologies*: <http://www.idtdna.com/scitools/applications/rnai/rnai.aspx>
- *GenScript*: http://www.genscript.com/siRNA_target_finder.html
- *Invitrogen*: <http://rnaidesigner.thermofisher.com/rnaiexpress/>
- *ThermoScientific*: <http://www.thermoscientificbio.com/design-center/>
- *InvivoGen*: <http://www.sirnawizard.com/construct.php>

³ We prefer using Invitrogen's RNAi designer as a tool to showcase shRNA design.

Materials:

- Computers with internet access OR BYOD with WiFi-capacity

Methods:

1. You will search for a particular mRNA sequence for a gene using available online software tools (using NCBI). Using one of these sequences, you will practice designing a siRNA for one of the candidate sites that you find.
 - **You will be looking for EGFP, enhanced green fluorescent protein = your target mRNA for knockdown⁴**
 - **Consult the e-Module 1 dealing with finding a nucleotide sequence for a particular gene (mRNA)⁵**
2. Follow the instructions of the TAs and coordinator. You will be asked to use one or more of the specified RNAi target site prediction tools above (e.g. Invitrogen's online application) to find candidate target sites for siRNA.
3. Note that to find EGFP – you have to do a *Nucleotide* search and **NOT** a *Gene* search.
 - **Consult the e-Module 2 dealing with siRNA target site determination⁴**
4. Save your results. Transfer some of the candidate sequences by copying-and-pasting into a Microsoft Word file.

OPTION 1: Part II – Bioinformatics - RNAi Primer Design*Goal:*

- *To design primers (specific nucleotide sequences of defined lengths) to create an RNAi construct (continued from Part I)*

In this and the subsequent sections, most of the information concerning siRNA design is derived from an article written by D. Castanotto et al. (2002). We invite you to read this article; they provide various PCR-based strategies to generate siRNA. We will be using one of these strategies for this lab but it is important to understand the other approaches as well.

In summary, you will be using a series of primers to generate a construct that will have all the components necessary to drive the autonomous expression of siRNA inside a mammalian cell. With the help of your primers and PCR, you will create a segment of DNA that will incorporate a human U6 RNA polymerase III promoter, sense and antisense RNA segments, a linker region (the hairpin loop) and a termination sequence for the RNA polymerase. There will also be some other modifications but that will be explained in the later sections.

N.B.: Primers are short oligonucleotide sequences that are complementary to specific regions in a nucleotide sequence. They are called “primers” because they “prime” or start the polymerase reaction which is critical in processes such DNA sequencing or the Polymerase Chain Reaction (PCR). We will be using the primers you design here in this section to create the shRNA-expressing construct by PCR later on.

The challenge at this point is learning what to do. Fortunately, as summarized above and in the literature, scientists have noticed certain structural and compositional features of effective siRNA. From their observations, we now have a set of guidelines to help design primers that can be used to make siRNA-expressing constructs.

You will select one of the candidate siRNA target sites from the previous section (Part I) and follow the instructions below to design primers that will allow you to generate a siRNA-producing construct.

It should be emphasized that this method is a fast and inexpensive way to allow scientists to generate siRNA-expressing constructs that can be screened to determine their effectiveness at gene silencing. This explains in part why we have chosen this method!

N.B.: The PCR product described in this section is an autonomous expression unit that can be transiently transfected directly into a eukaryotic cell line to knock-down gene expression.

Materials:

- Results from Bioinformatics Part I
- Computer with internet connection
- Web browser with latest drivers

⁴ A sample of the kind of sequence students should find is provided in APPENDIX B.

⁵ It is recommended that video teaching aids (not provided here) be used to complement the text.

Rationale:

The procedure for the PCR approach is depicted in Figure 1. Like all PCR reactions you will need primers and a template. Here the template is the human U6 RNA Polymerase III promoter (U6 Pr). The primers will vary depending on the method used.

Again, you will be designing primers that will allow you to generate a DNA construct by PCR that has all the components necessary to generate siRNA (Figure 1D).

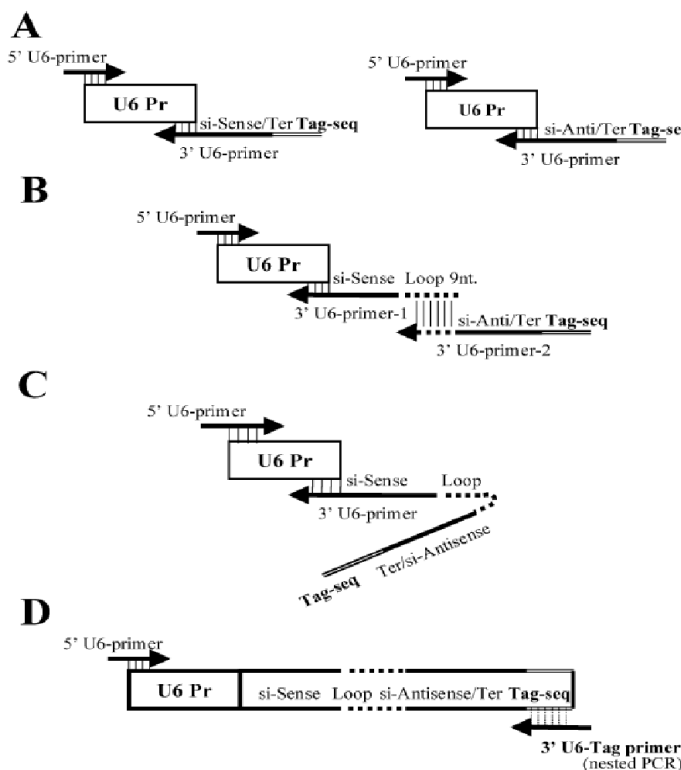


Figure 1. PCR-based siRNA design (from Castanotto et al., 2002)

as shown in Figure 1D although 1B requires 2 rounds of PCR while 1D requires only 1 round to create Figure 1D. Again, Figure 1D is an autonomous shRNA-expression element; it can be purified and then transfected into a eukaryotic cell line to inhibit expression of the target.

Methods:

Go to NCBI the website (<http://www.ncbi.nlm.nih.gov/>) and find the human U6 RNA pol III promoter sequence.

1. Since we need U6 specific primers in all 3 scenarios (Figure 1), we will need the sequence of human U6 RNA polymerase III.⁶ **Using NCBI website, find the complete DNA sequence for this cis-regulatory element.** Using the sequence, follow the steps below to design the upstream FORWARD U6 primer & REVERSE U6 primer.

N.B.: For the purposes of record-keeping and transparency, all primer designs are recorded in a text-processing file (e.g. Microsoft Word).

2. We will be designing primers that allow us to isolate the COMPLETE U6 RNA pol III promoter. So that means the FORWARD primer and REVERSE primer are on either end.

Since we are trying to generate siRNA, it not surprising that we need a promoter for a RNA polymerase in our PCR product. As it turns out, the RNA polymerase III promoters (ex. human U6 promoter) are very effective in mammals to generate siRNAs (Miyagishi and Taira, 2002). This is why all the panels in Figure 1 show the U6 promoter (U6 Pr) upstream of the siRNA; this promoter is the entry site for RNA pol III to start transcription.

All RNA polymerases need a termination sequence to tell it to stop transcribing. The RNA polymerase III termination sequence is a stretch of 6 deoxyadenosines (Ter). Ignore the “Tag” sequence (Figure 1) for the moment (it includes a restriction enzyme site).

Figure 1A shows how to make the sense and antisense siRNA independently. By contrast Figures 1B and C allow us to make a construct with the sense and antisense expressed together, linked by a 9 nucleotide linker (loop) sequence. This type of construct will generate short hairpin RNA (shRNA) that will then be processed into siRNA in the cell.

Notice that depending on the method used, the number and type of primers will vary. Both Figure 1B and 1C will generate a construct

⁶ See APPENDIX B for the target sequence that students need to find.

- Remember the orientation and sense of your primers. The FORWARD primer will be upstream pointing down the sequence 5'-to-3'; its sequence is based off the sense strand of the U6 sequence. The REVERSE primer will be at the other end, pointing back up the sequence 5'-to-3'; its sequence is complementary (antisense) to the U6 sequence found through NCBI.
- There are certain **guidelines** that should be abided (if possible) when designing primers:
 - GC content should not exceed 50% of the primer when possible
 - 3' end of the primer should end in at least two G's and/or C's.
 - The melting temperature of your primer (T_m) should be between 55-65°C; the T_m of the two primers should not differ by more than 3°C
 - Related to the T_m, the PCR primers are usually no shorter than 15 nucleotides
 - REMEMBER THESE ARE JUST GUIDELINES!⁷
- Melting Temperature (T_m)** refers to the temperature at which 50% of a population of primers remain annealed to their template; increasing the temperature results in a greater % melting off the template sequence. This T_m is important when we do our PCR reactions with your primers. There are different formulas that can be used to measure T_m and some are more accurate than others. Here is the simplest but least accurate formula:

$$T_m = 4^\circ\text{C}(\# \text{ of G/C}) + 2^\circ\text{C}(\# \text{ of A/T}) \quad [1x \text{ SSC conditions}]$$

$$\text{Eg. Primer } X = 5'\text{-AATCCGGAAACCGGATT-3'} \text{ has a } T_m = 4(8 \text{ G/C's}) + 2(9 \text{ A/T's}) = 50^\circ\text{C}$$

- Use this formula for the moment to design your primers. It should be noted however that you will be using some software to calculate the T_m that employ a different formula (see Primer Design Module). The T_m may change and so may your primers. So long as your T_m falls within the 55-65°C range, you are in good shape. Primers whose T_m is too high are problematic and expensive to manufacture. Primers whose T_m is too low are cheap to make but inefficient in use and may work in ways that are not ideal (ex. Non-specificity).
- It is preferable that the primer contains at least 2 G or C residues (GG, GC, CG, CC) at its 3' end because, unlike A/T, G/C form greater hydrogen bonding. Given that the polymerization of nucleotides begins at the 3' hydroxyl on the end of the primer, the 3' end is the most important end on the primer; it must be annealed to the template sequence for the polymerase to start DNA replication. That being said, too many G/C residues increase the chances of stable hairpin and primer dimers. Ideally, G/C composition of a primer sequence should not exceed 50%.

****AGAIN, THESE ARE GUIDELINES AND GIVEN YOUR SITUATION, DO NOT BE TOO CONCERNED IF YOU CANNOT SATISFY THEM ALL! AT MINIMUM, MAKE THE T_m OF YOUR PRIMERS AS EQUAL AS POSSIBLE. ****

- That being said, we can check these primers for any issues. Go to this website <https://www.idtdna.com/calc/analyzer> and you should see something like this (Figure 2). Notice the list of options on the right. Do “Analyze”, “Hairpin”, and “Self-Dimer”. Are there any problems? If so, should we be worried about it?

Figure 2. IDT's OligoAnalyzer opening page

⁷ Some guidelines may be broken due to constraints imposed by your gene sequence. The goal here is to be aware of possible explanations as to why your primers may not work later.

9. Repeat with each other primer.
10. We can also check for hetero-dimers, i.e. your FWD binding to your REV primers. Click on “Hetero-Dimer” and enter both primer sequences in their respective boxes. Do the comparison.

****AGAIN, THERE MAY BE NOTHING YOU CAN DO TO FIX ANY POTENTIAL PROBLEMS WITH YOUR PRIMER DESIGNS (DEPENDING ON THE SITUATION). IF HOWEVER YOU CAN MAKE CHANGES TO THE SEQUENCE TO REMOVE ANY ISSUES (EX. HAIRPINNING OR DIMERIZATION), FIRST ASK US WHY OR WHY NOT.****

11. Now that is over with, you now need to ensure specificity of your primer designs (go to the next section, Bioinformatics Part III). Make sure they can bind to U6 RNA pol III. Then come back here and continue on.
 12. Once the specificity has been confirmed, we can continue. Of the 3 methods shown in Figure 1 above, we will be doing Figure 1C. Now that you have designed the FORWARD and REVERSE U6 primers, we now need to modify our primers, specifically the primer that will incorporate both the sense and antisense sequences to create a shRNA. Based on Figure 1C, we see that the REVERSE U6 primer will carry the sense-loop-antisense-termination sequences at the 5' end of the REVERSE primer.
 13. Notice the order of these elements in the REVERSE primer of Figure 1C. The 5' end will start with the Termination sequence followed by the antisense sequence, followed by the linker sequence, followed by the sense sequence, and finally by the U6 promoter sequence at the 3' end.
 14. Also remember to add deoxyribonucleotides to your sequence (no uracils). I know this can be confusing since we are making siRNA but PCR does not amplify RNA! YOUR PRIMERS ARE IN DNA!
 15. Also another final complication – remember that the REVERSE primer is the reverse complement (i.e. antisense) of the sense strand. So when you add nucleotides, you must add the reverse complement sequence in the proper direction, 5'-to-3' (ex. If the sense strand is 5'TCCG3' then the antisense becomes 5'CGGA3')
 16. Remember that the linker sequence is a 9 nucleotide stretch, determined to be on average the best length for a hairpin loop (Castanotto *et al.*, 2002). Moreover, avoid adding a stretch of 4 or more T's in the sense strand in the loop.
 17. The *Termination* sequence is a stretch of 6 T's (on the sense strand sequence).
 18. Make these modifications to your REVERSE primer and once you are done, show your design to the TAs or course coordinator to confirm proper design.
- N.B.: Here concludes Option 1 (the fastest and least costly method at producing a shRNA-expression construct). These primers can be ordered and used in a PCR reaction to create a shRNA-producing gene that can then be transfected into cells to knockdown expression (see Part VI). Please note however that a non-specific control shRNA will be needed as well (see Part V, Step 9).**
19. If you wish to insert the shRNA-construct into a plasmid (e.g. for GFP-tagging your transfected cells), you may now continue to optional Bioinformatics Parts IV and V (Option 2).

OPTION 1: Part III – Bioinformatics - Testing the Specificity of Your Primers

Goal:

- *To assure that the primer you designed can “find” your target sequence.*

You will now test to make sure that your primers from Section II are truly specific for the DNA element you are trying to copy and amplify. **This is especially important for cloning purposes because we want your primers to target only the desired gene for cloning and nothing else. We can use the same application (Oligoanalyzer) from Section II that checked the quality of your primers.** This same software will take the sequences of your primers and test them for possible matches to other sequences in a vast database (GenBank). The identity of possible matches will be displayed along with information about the degree and location of the match. In the end, you may find that your primer can recognize gene sequences in addition to U6. These other matches may pose a problem in your experiments but we can discuss this further in the lab.

Materials:

- Computer with internet access
- Web browser
- Your primer sequences

Methods:

1. Make sure you look at e-Module 3 first to see the steps for this section.
2. Using OligoAnalyzer, you can now select the “NCBI Blast” option in the list on the right.
3. This should take you to a new page that looks something like this (Figure 3).
4. You will notice that your primer sequence has been pre-loaded in the large box near the top.
5. Since we are interested in human genes, we want to only check specificity against human sequences.⁸ Just below is an item called **Database** and it is defaulted to look at “**Nucleotide collection (nr/nt).**” Change it to “**Human Genomic plus transcript (Human G+T).**” Your primer will be tested against all known HUMAN gene transcripts.
6. Select “BLAST” just below.
7. In a few moments, you should see a new window in which you will see if your primer can find your gene as well as other genes you do not want (Figure 4).
8. You now have a list of sequences (not necessarily genes) that contain identical or near-identical sequences to your primer. The degree of identity between your primer sequence and the possible target will depend on parameters that were set in the application (we use the default settings). These parameters may be changed, however, and will have an effect on the results.
9. You will see that that there is more than one possible match for your primer sequence meaning that your primer could inadvertently recognize something other than what you want.
10. You wish to confirm that your primer **completely** matches your **desired** target. Therefore, if you designed primers specific for the mouse CD8 gene, for example, the match should appear 100% for mouse CD8 for both primers (i.e. every nucleotide in your primer corresponds to an identical nucleotide in the target sequence).
11. Do not be surprised however if your primer binds to other sequences. This is especially true if the gene is highly conserved and/or part of a family of genes (e.g. immunoglobulin domain in Ig superfamily). If so, this could be a problem.
12. Once you know that your primers are specific to your gene-of-interest, you can use these primers to isolate and amplify your target gene from DNA of cells known to contain the gene. The process we will be using to carry out this gene-specific amplification is called **Polymerase Chain Reaction (PCR)**.
13. Return to Step 11 in Part II above and continue.



Figure 3. NCBI’s BLAST opening window.

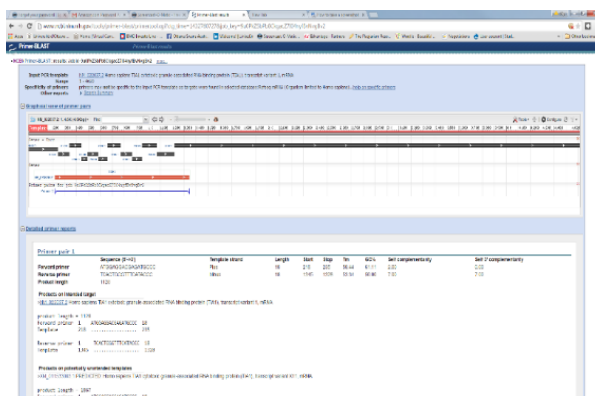


Figure 4. NCBI’s BLAST sample search results.

⁸ The criteria of the BLAST search will change depending on the needs of the course and the nature of the sequence being tested.

OPTION 2: Part IV – Bioinformatics - Restriction Mapping of Your Construct

Goals:

- For the option of cloning the PCR product into a plasmid (e.g. for GFP-tagging cells with shRNA).
- To generate a restriction enzyme map of your construct.
- To determine where, what, and how many times a library of known restriction enzymes do or do not cut your construct.

Materials:

- A computer with internet access
- A web browser with updated drivers
- Your complete construct sequence including U6 with the shRNA tail

Methods:

You will be creating a *Restriction Map* of your construct. In brief, restriction enzymes (r.e.) are endonucleases that recognize palindromic dsDNA sequences and cleave dsDNA within these sites. Different enzymes recognize different palindromes. However, some enzymes do share the same palindrome although they may cleave the dsDNA at different sites within the palindrome.

Software is available free-of-charge on the web that can survey a sequence for the different palindromes that correspond to different restriction enzymes. These “restriction enzyme sites” are then mapped along with the identity of the enzyme that recognizes it.

1. Log on to your computer and access a web browser.
2. Go to this web address: <http://tools.neb.com/NEBcutter2/index.php>. IF THIS ADDRESS DOES NOT WORK, GOTO www.neb.com AND LOOK FOR *NEB CUTTER*.
3. You should come to a webpage that looks like Figure 4.
4. Follow the instructions on the webpage. Cut and paste your DNA construct sequence (no numbers – text only) into the large white box.
5. Click “SUBMIT”.
6. You will be taken to a new webpage where your sequence is displayed along with the position of different restriction enzyme sites, where they cut, how many times, etc. (Figure 5)
7. Click on the “0 Cutters” (circled) to see the list of enzymes that DO NOT CUT your construct sequence.
8. Save your output. If you can, make a print-out.
9. With this information, you can go on to Bioinformatics Part V.

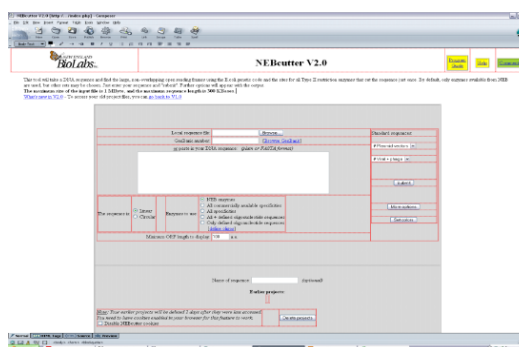


Figure 4. New England Biolab’s NEBcutter opening window.

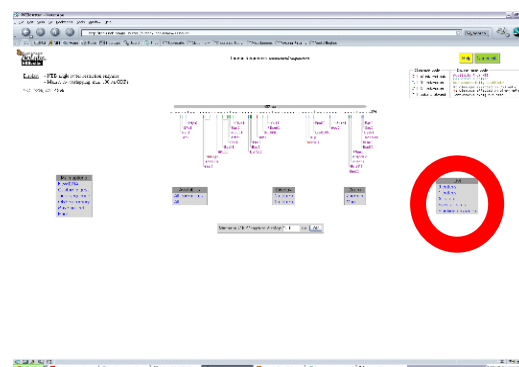


Figure 5. New England Biolab’s NEBcutter restriction mapping results.

OPTION 2: Part V – Bioinformatics - Adding R.E. Sites to Your Primers*Goal:*

- To introduce restriction enzyme sites at the 5' ends of your primers for the purposes of inserting a construct into a DNA plasmid (continuing from Part IV).

Materials:

- Your primer sequences
- Your restriction map of the target construct (from Bioinformatics Part IV)
- Restriction map of the different vectors (see below)

Methods:

- You will incorporate restriction enzyme sites on to the 5' ends of your primers. These sites will make it convenient to insert the construct into vectors for the purposes of i) large scale replication and ii) expression studies (to be done later).
- R.E. sites to be added to your oligonucleotides **CANNOT** be found in your construct but must be found in the expression vector, in this case, pEGFP-C3 (see map below). We have included a copy of the map for both plasmids including their multiple cloning sites or regions (MCS or MCR).
- Below are the maps for the two vectors you will be using in this course (Figure 6). Note how they highlight their MCR regions, usually by listing different r.e. sites. In our case we will focus on pEGFP-C3, not pDrive (to be explained later).

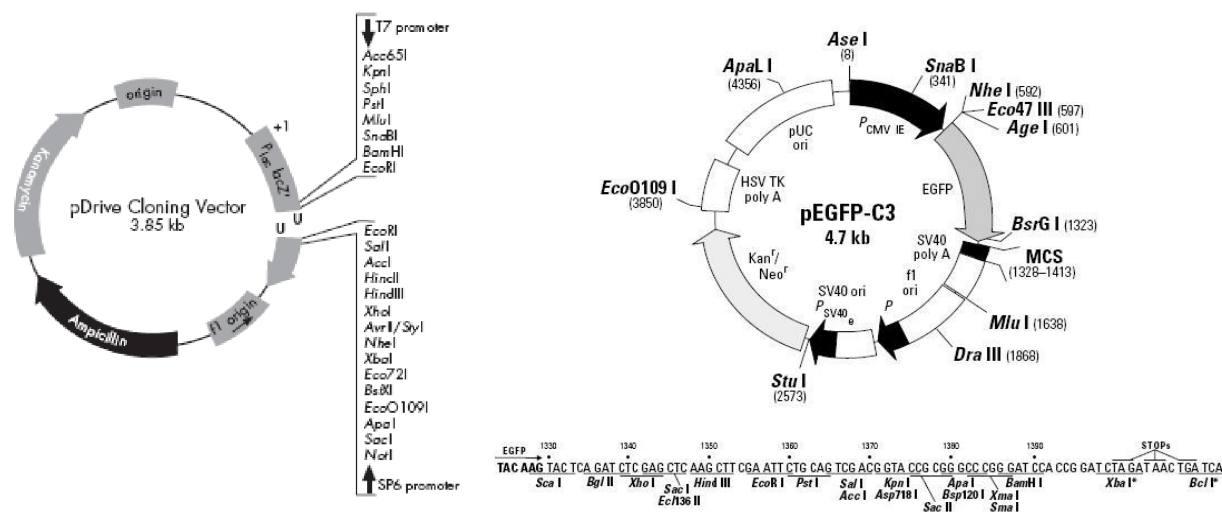


Figure 6. Maps for pDrive (left) and pEGFP-C3 (right). The pDrive plasmid (Qiagen) is used for subcloning of the shRNA-expressing construct from Parts I to III before final insertion into pEGFP-C3. The EGFP in the latter allows for fluorescent tagging of transfected cells (see Part VI).

- The matches represent enzymes that you can use for the following steps.
- As indicated earlier, you are going to add r.e. sites to the **5' end** of your primers (see figure below). Again, these r.e. sites are present in the vector pEGFP-C3 but **NOT** in your construct (“0 cutters”). Since you have designed only **TWO** primers, you will add only **TWO** r.e. sites; the r.e. sites may be the same or different, depending on the situation. The challenge is to figure out which enzyme(s) you wish to use.
- You need to be mindful of which enzyme(s) you decide to use. As shown above, you will add the corresponding palindrome sequence for each r.e. at the 5' end of each primer. If you are adding two different r.e.'s sites, read the following. If you are adding only one r.e. site to both ends, skip to step 8.

- a. First, choose two enzymes that can use the same r.e. buffer; choose a buffer that gives 100% digestion for both enzymes (see APPENDIX A6, R.E. Buffer Chart). Using the same buffer saves considerable time.
 - b. Next, the forward primer will receive the r.e. site that is upstream of the other r.e. site in the DNA sequence of pEGFP-C3. *eg. If you choose Bgl II and Pst I, Bgl II is upstream of Pst I in the MCR of pEGFP-C3 (see MCR above). Therefore, the **forward primer** will get Bgl II 's r.e. site added to its 5' end. The **reverse primer** will receive the Pst I palindrome.*
 - c. Last, choose two enzymes that are separated by a minimum of 6 nucleotides in the sequence of pEGFP-C3.
7. Make sure that your palindrome identity is maintained, 5'-to-3', after addition to the 5' end of your primers.
Ex. If the palindrome is 5'GGATCC3', then you add it in that EXACT sequence to the 5' end of the primer.
 8. These primers are now ready to be used in a Polymerase Chain Reaction (PCR) to amplify DNA that contains all the elements needed to express your shRNA. With the restriction enzyme sites at both ends of your amplified shRNA-expressing DNA construct, you can insert it into a plasmid by restriction digest and ligation. This way, you will be generating a plasmid-based shRNA expression construct that also co-expresses EGFP (see APPENDIX B for examples).
 9. A non-specific shRNA-expressing construct will also be needed. This construct follows all the steps described above but differs from the specific version at its sense and antisense regions (Figure 1). The normal protocol is to make a copy of the gene-specific REV primer from Figure 1C and then randomize its sense and antisense sequences only. Be sure that the randomized sequences are complementary, however. Otherwise, all the other components of the constructs are maintained. This randomized version should not target the specific transcript and serve to control for non-specific effects with shRNA expression.

Part VI – Measuring the Knockdown Effect with shRNA

With the primers now designed (either Option 1 or 2), these can be ordered online (e.g. through Invitrogen) and delivered to the lab usually within a week. With these primers in hand and using a DNA template bearing the U6 promoter, two separate PCR reactions (one for the non-specific control, another, specific) can be performed by the class. The PCR products can be separated on an ethidium bromide (EtBr)-agarose gel and visualized with a UV box to confirm successful production and for purification purposes (e.g. using DNA-gel purification kits from Qiagen). The clean DNA can then be used in a transfection assay using any number of Biosafety Level 1 (BSL1) compliant eukaryotic cells (e.g. P19, C2C12, or CHO) and a commercial transfection reagent, such as Lipofectamine[™] (ThermoFisher) or GeneJuice[™] (EMDMillipore). Alternatively, the constructs can be inserted into a reporter vector such as pEGFP-C3 before transfection. In so doing, students can track cells transfected by their shRNA-expressing constructs through the use of the fluorescent reporter gene.⁹

After transfection, RNA can be extracted and reverse transcribed with the help of an oligodT primer to generate a mixed cDNA population. Since it is assumed that a PCR machine is available to create the expression constructs in the first place, this same machine can be adapted for use in a non-competitive reverse transcription (RT-) PCR assay (Gause & Adamovicz, 1994) in order to measure the level of knock down of the targeted transcript by the shRNA. For this RT-PCR, two cDNA transcripts need to be examined, namely the target (e.g. EGFP in this report) and a housekeeping gene (e.g. GAPDH). In short, students perform separate PCR reactions, one for each cDNA, amplified over various cycles, load the reactions on an EtBr-agarose gel and migrate, view the PCR products on a UV box, acquire a digital image, and perform densitometry analyses of the band intensities (e.g. with free software such as ImageJ¹⁰). A semi-quantitative analysis is therefore possible to compare and contrast the shRNA's suppressive effects of the specific shRNA versus the non-specific variant at various time points. For details as to how to do this, refer to Gause & Adamovicz (1994). Keep in mind that this is but one way to measure the knockdown effect. Other approaches may be exploited depending on the nature and function of the gene being targeted as well as the equipment on hand. Depending on the situation (i.e. target gene and resources available), the specific approaches will be added to the lab manual for the students to follow when and where appropriate.

⁹ This ability to track fluorescence is not necessary function for the shRNA to knockdown its target and is only important if your target is EGFP (which it is in this report) and/or your teaching lab is equipped to measure fluorescence.

¹⁰ <https://imagej.nih.gov/ij/>

Materials

- Parts I to V – Bioinformatics Portion involving design of shRNA-expressing construct
 - Digital devices with internet connection
- Part VI – Making and Testing shRNA
 - Ability to synthesize in-house or order oligonucleotides from Parts I to V for synthesis (e.g. from Life Technologies Inc.)
 - PCR machine and reagents for PCR reaction including:
 - dNTPs, oligonucleotides from Parts I to V (primers), a U6 RNA polymerase II template (human), DNA polymerase, DNA polymerase buffer (Taq)
 - reverse transcriptase (MMLV), RT buffer, and oligo dT
 - Agarose gel apparatus with power pack and suitable migration buffer
 - Agarose gel with ethidium bromide
 - UV box for gel imaging
 - Digital documentation device for image taking of gels
 - Image analysis software for densitometry of bands (e.g. free ImageJ)
 - DNA isolation kit from agarose gel (Qiagen)
 - Biosafety Level 1 (BSL1) mammalian cell line (e.g. CHO or P19)
 - Tissue culture facilities (e.g. incubator with CO₂) and reagents to maintain cell lines
 - Transfection Reagent (Lipofectamine[™], GeneJuice[™] or the like)
 - Total RNA isolation kit from cell lines (Qiagen)
 - **Optional:** Competent recA- bacteria (e.g. DH5 α , HB101, etc.), LB-Agar plates with antibiotics, incubator
 - **Optional:** DNA isolation from bacteria cultures (miniprep; Qiagen)
 - **Optional:** UA cloning (pDrive; Qiagen) and reporter plasmid (pEGFP-C3; Clontech)
 - **Optional:** Restriction enzymes and buffer (e.g. MluI; NEB)

- **Optional:** Fluorescence Microscope/ Plate Reader/ Flow Cytometer

Notes for the Instructor

These notes provide some important insight and context that the reader may find useful either before or after reading the student protocols above. In short, Parts I to V are copied from the lab exercises for Days 1 and 2 (Bioinformatics, APPENDIX A) of the actual lab course. They describe how students can design short DNA oligonucleotide sequences that after PCR will contain all the elements necessary to produce shRNA in a transfected mammalian cell line. This PCR-based strategy is derived from the foundational work of Castanotto, Li, and Rossi (2002 – a must read), who describe how to design and make a shRNA gene under the control of the human U6 RNA polymerase II promoter. This cis-acting element serves as the template for the PCR reaction with oligonucleotides designed from Parts I to V above. Integrated into the shRNA design process is the optional 5' addition of restriction enzyme sites (APPENDIX B) allowing the insertion of the PCR product into an EGFP expression vector, namely pEGFP-C3 (Days 3 to 14, APPENDIX 1). The pEGFP-C3 provides the user the opportunity to fluorescently tag those cells transfected with the shRNA construct. For the purposes of this report, the pEGFP-C3 vector is critical since EGFP mRNA is also the shRNA target and mammalian cells normally do not express this xenogene. Consequently, it must be co-inserted ectopically with the shRNA construct in order to see any effects. After making two different recombinants of this modified vector, one with a non-specific shRNA sequence and another with the EGFP-specific version, students can measure and compare the changes in EGFP expression (either at the mRNA level or at the protein/function level) at various time points post-transfection (Part VI). In so doing, students can assess the specificity and effectiveness of the RNAi-mediated 'knockdown' as described in the literature.

Here are some additional points that any lab instructor wishing to add shRNA to their lab may need to consider in the planning process:

- As outlined in protocols I to V of this report (see below), free online software suites are used extensively in the design of the DNA oligonucleotides including the (optional) addition of restriction enzyme sites for cloning the construct into an expression vector.
 - Students will need internet-enabled devices to carry out these tasks, either

- with their own or with institutional resources.
- Normally I to V can be accomplished over the course of three to five hours of supervised class time.
 - **OPTION:** If the instructor does not wish to insert the shRNA construct into a vector (e.g. pEGFP-C3), protocols IV and V can be skipped.
 - Two sequences are needed, namely the complete human U6 RNA polymerase III promoter and the target gene sequence (in this example, EGFP) (see APPENDIX B).
 - Students will need to design oligonucleotides that can be used by PCR to produce either a nonspecific control or the EGFP-specific shRNA construct.
 - It is recommended that all the students start with specific construct.
 - Non-specific controls are randomized sequences of the specific shRNA elements.
 - This approach requires access to a PCR machine and associated reagents.
 - A vector containing the human U6 RNA polymerase III promoter is necessary to serve as the template for the initial PCR.
 - **OPTION:** The students can follow the steps outlined in the protocols to design their own sets of oligonucleotides but it is logistically simpler, cheaper, and pedagogically sound to provide students with pre-designed primer sets once the bioinformatics exercise is completed. In this course, odd groups work with the non-specific shRNA construct while the even groups work with the gene-specific variant.
 - If cloning of the shRNA construct in a vector is desired, be mindful where it is to be placed. In this case, the shRNA can NEVER be placed in the traditional insertion locale that is the multiple cloning site (MCS) because it will inadvertently destroy the mRNA in a non-specific manner. For this reason, MluI was used for insertion of the shRNA PCR product into pEGFP-C3.
 - From APPENDIX A, the reader will notice that a UA cloning vector is used (pDrive). Students first subclone into pDrive before transferring the PCR construct into pEGFP-C3. Again this subcloning step is optional.
 - The insertion into an expression vector is optional. That being said, transfecting a **reporter vector is highly recommended** to measure transfection efficiency in the cells.
 - **OPTION:** The PCR product (after agarose gel purification) is capable of knocking down expression (cheapest and fastest alternative) if directly transfected into cells (no vector insertion is required).
 - **OPTION:** A vector like pEGFP-C3 can be **co-transfected** with the purified PCR product as two separate entities.
 - Any gene transcript can be targeted by this methodology. That being said, it is ideal to have multiple gene candidates, especially from published studies that provide evidence of working siRNA or shRNA sequences (e.g. the EGFP shRNA in APPENDIX B).
 - The software suites showcased in Part I are predictive tools but it may be preferable to go with published sequences that are known to work.
 - More gene candidates means that you can change the course year-after-year to minimize the possibility of students profiting from previous cohorts. Aim for at least six, one per iteration of the course.
 - Endogenous gene targets in a cell require high transfection efficiency (>90%) to “see” a knockdown effect. If the transfection efficiency of your cells falls below this threshold, it is advisable to switch to a non-endogenous (xenogenous) target gene (see next item).
 - Based on experience, it is preferable to co-transfect two exogenous gene

constructs, one for the shRNA, the other for a gene not normally expressed by the transfected mammalian cell. For example, GFP is normally expressed in *Aequorea Victoria* and will not be expressed in a mammalian cell line. Here, high transfection efficiency is not as critical to see a ‘silencing’ effect.

- Knockdown takes time. Be prepared to wait 24 to 72 hours post transfection to see a reduction in expression (see Link to Supplemental Files).¹¹
 - One must consider biosafety restrictions and provide the appropriate equipment when working with cells. Ideally, biological agents, such as the cell lines, should not exceed **Biosafety Level 1** workplace conditions.
 - This may be the most cost prohibitive aspect of the course (see APPENDIX C), namely requiring cell culture and transfection reagents.
 - Additionally, DNA used to transfect cells must be from a clean preparation (e.g. Qiagen miniprep kits for DNA isolated from bacteria preparations).
 - If reverse transcription (RT)-PCR is planned, a suitable protocol for RNA extraction from transfected mammalian cells will be needed.
- Assays that measure the knockdown effect (part VI) can include but are not limited to:
 - Noncompetitive RT-PCR, qRT-PCR, and Northern (to measure mRNA knockdown directly), and
 - Fluorescence-based, chemiluminescent-based, and absorbance-based assays (to measure specific gene function alterations).
 - Again, endogenous targets in the host cell line will require high transfection efficiencies to detect any significant changes in expression and/or function.
 - Xenogenic targets (e.g. EGFP in mammals) are more forgiving from an experimental standpoint and provide as

valid a learning experience as endogenous targets.

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Professor Marc-André Langlois (University of Ottawa) for providing the initial U6-bearing shRNA construct (as a gift for teaching purposes) and expertise in making this project possible. Lucille Joly for excellent technical work at making and pre-testing each construct before class as well as setting up equipment and reagents for each class.

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¹¹ Update as of 2016: We saw reduction by RT-PCR and fluorescence microscopy by 16 hours post-transfection in C2C12 cells with EGFP-specific shRNA.

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Appendix A Sample Course Outline

Here is a copy of the Table of Contents for BPS4127 (Fall 2013) in which students created an shRNA against the EGFP transcript. This list outlines the order and types of experiments done over a 12-week semester with two 3-hour lab sessions or *days* per week. As outlined in the introduction, the general order and steps remain the same year-after-year but the targeted transcript changes with each iteration. For example, in 2013, EGFP was the target (as described in this report) while, in 2014, Bmi-1 was selected. With 5-6 different targets, a rotation of projects can be created.

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COURSE BACKGROUND

- i. - Who we are:
- ii. - Course Objectives:
- iii. - Required Material:
- iv. - Assessing Marks:

Day 1- Introduction to BPS4127 and Bioinformatics, Sections 1.1 to 1.3

- 1.0 - Introductory Laboratory
- 1.1 - Bioinformatics Part I – RNAi 101
- 1.2 - Bioinformatics Part II – Primer Design
- 1.3 - Bioinformatics Part III – Testing The Specificity of Your Primers

Day 2- Bioinformatics continued, Sections 2.1 to 2.2

- 2.1 - Bioinformatics Part IV – Restriction Mapping of Your Gene
- 2.2 - Bioinformatics Part V – Adding R.E. Sites To Your Primers

Day 3 – PCR Optimization, and Vector Prep I

- 3.1 - Polymerase Chain Reaction: DNA to more DNA
- 3.2 - PCR: Test primers under different conditions
- 3.3 - Restriction enzyme-mediated digestion of vector DNA
- 3.4 - Vector Preparation Part I

Day 4 – PCR product purification; subcloning into pDrive

- 4.1 – Purification of PCR product
- 4.2 - Purification of DNA from an agarose gel
- 4.3 - Cloning into pDrive vector (SUBCLONING)

Day 5 – Transformation and Vector Prep Part II

- 5.1 - Transformation of Bacteria with DNA plasmid
- 5.2 - Part II – Dephosphorylation/purification of Vector DNA

Day 6 – Journal Club 1

- 6.1 – Journal Club 1

Day 7 – Minipreps and R.E. Digests

- 7.1 - Plasmid DNA Boil Prep protocol
- 7.2 - Digestion of your DNA preps with R.E.

Day 8 – Identification of prep with insert; scientific paper writing

- 8.1 - Analytical gel your *digested* construct and large digest

Day 9 – Purification of the construct from pDrive, quantification of vector and insert, ligation into pEGFP-C3

- 9.1 - Purification of DNA from an agarose gel slice
- 11.1 - Ligation of Digested DNA into Vector

Day 10 – Midterm

Day 11 – Transformation and Journal Club 2

- 11.1 - Transformation of Bacteria with DNA plasmid
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Day 12 – PCR Screening of Colonies. Flow Cytometry lecture

- 12.1 – PCR – Screening of Bacteria Colonies for Your Construct
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Day 13 – Gel of PCR ,Clean DNA Miniprep and Flow Cytometry Data Analysis

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APPENDICES

Appendix A1 - Making Agarose Gels

Appendix A2.1 - Quantifying your RNA

Appendix A2.2 – Quantifying your DNA

Appendix A3 – Buffer Recipes

Appendix A4 –DNA Ladder

Appendix A5 – Quantification of DNA on a gel

Appendix A6 – R.E. Buffer Chart

Appendix A7 – Writing a Scientific Paper

Appendix A8 – Preparing Primers

Appendix B Sample Sequences

Here are the accession numbers for both the U6 human RNA promoter and the EGFP gene. The former is necessary to design the U6-specific portion of the primers used to create and amplify the shRNA-expressing DNA construct. The EGFP sequence is needed to create shRNA elements (sense and antisense) that will be incorporated in the reverse primer (see Figure 1, Part I). In so doing, the shRNA will target and knock down EGFP expression. Andreou et al. (2003) was used as the source of EGFP-specific shRNA sequences (GFP22-siRNA, Qiagen). Ideally, it is preferable to use published sequences from studies that show a reduction effect rather than sequences suggested by the online tools.

- **Homo sapiens U6 snRNA gene, complete sequence** GenBank: JN255693.1
- **Lentiviral vector pDG2i-eGFP-V5-Puro, complete eGFP sequence** GenBank: LT009443.1

Here are the oligonucleotide (primer) sequences used in this iteration of the project. The forward primer is used with either reverse primer to generate a shRNA-expressing construct. For the PCR experiment that will allow a student to make the construct, a template containing the complete human U6 RNA polymerase III sequence is needed. Included in these sequences are restriction enzyme sites used for insertion of the PCR products into an expression vector (OPTION 2).

U6 FORWARD Oligonucleotide

- 5'-ACGCGT CACAAGGTG GGATCC AAGGTCGGGCAGGAAGAGGGCCT-3'

Mlu I – Dra III – BamH I – U6 promoter sequence

EGFP-specific shRNA REVERSE Oligonucleotide

- 5' ACGCGT CACAAGGTG AAAAAA CGGCAAGCTGACCCTGAAGTTCATCGTCTTGAA ATGAACTTCAGGG TCAGCTTGCGGTGTTCGTCCTTCCAC-3'

Non-specific shRNA REVERSE Oligonucleotide¹²

- 5' ACGCGT CACAAGGTG AAAAAA CGGACTTCATAAGGCGCATGCCGTCCTTGAA GCATGCGCCTTATGAA GTCGGTGTTCGTCCTTCCAC-3'

Mlu I – Dra III – Terminator – CG – antisense sequence – loop – sense sequence – U6 promoter sequence

¹² This non-specific primer is not a randomized variant of the EGFP specific variant. That being said, it was a negative control derived from a previous publication (Jiang et al., 2010) and did not affect EGFP expression.

Appendix C Expenses

This is a partial list of the costs associated with the lab. It does not include all the consumables and assumes certain equipment already being available (e.g. pipettors, pipettes, tips, agarose gel apparatus, power packs, PCR machine, etc.). As of 2016, this lab is assigned a course budget of Cdn\$5800 for consumables for 3 sections of up to 32 students per section. **Items in bold are optional.**

Line #	Item	Company	Cost per Unit (Cdn\$) ^a
1	Primers for PCR ^b	Life Technologies Inc.	\$11-\$25 per primer
2	Mammalian Cell-line (BSL1) eg. CHO ^c	Sigma-Aldrich	\$495.00 ^d
3	Tissue Culture 6 well plates (50 plates)	VWR International	\$68.77
4	Genejuice Transfection Reagent (1 ml)	Fisher Scientific	\$420.00
5	Eukaryotic expression vector (pEGFP-C3)^e	Clontech	~\$700
6	DNA Gel Extraction Kit (for 50 samples) ^f	Qiagen	\$104.95
7	Mlu I (5000 Units) (1 vial)^g	New England Biolabs	\$296.65
8	UA DNA cloning Kit (for 40 samples)^h	Qiagen	\$360.00
9	Bacteria for DNA Transformations (DH5α)	ThermoFisher Scientific	\$238.35
10	Small-scale DNA Isolation Kit (50 samples)ⁱ	Qiagen	\$70.70
11	RNeasy Plus Mini Kit (50 samples) ^j	Qiagen	\$400.00
		Actual Total (with Optional items)	~\$1563.72 (~ \$3229.42)

- a. The listed costs are in Cdn\$ and are pre-tax for a single item. Costs are as of the summer of 2016.
- b. Given that this protocol relies on PCR, one assumes that the equipment and consumables necessary for PCR are available. For primer sequences, see APPENDIX B.
- c. One assumes that the equipment and consumables necessary for maintaining a mammalian cell line are available. Chinese Hamster Ovarian cells are provided as an example of a Biosafety Level (BSL) 1 approved cell line.
- d. Values in brackets denote non-mandatory costs. For example, these cells were received for free for education reasons thanks to kind donations from in-house research labs.
- e. Any eukaryotic expression vector (ideally with a reporter molecule such as EGFP) can be used. This particular vector was received as a gift but can be purchased if necessary. Although recommended, **this addition is optional.**
- f. Gel extraction is necessary for PCR product purification for either transfection or **optional cloning into a vector.**
- g. The PCR product was cloned into pEGFP-C3 at the Mlu I site (position 1638). **This step is optional.**
- h. For cloning purposes, the PCR product is subcloned into pDrive before transfer to pEGFP-C3. **This step is optional.**
- i. If the construct is cloned into an expression vector, this step must be included before transfection.
- j. This is necessary to extract RNA for the RT-PCR analysis to measure reduction of targeted mRNA.