**RNA Aptamer Selection against Bcl-2 to Promote Apoptosis in Cancer Cells**

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Pool R50, RNA, BCL-2

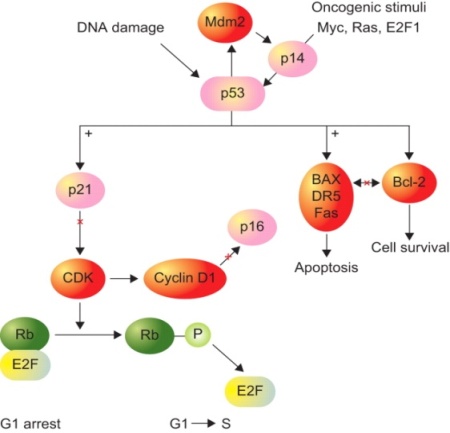
**Abstract**

B-cell lymphoma 2 (Bcl-2) is an integral membrane protein found on the outer membrane of mitochondria, the intermembrane of endoplasmic reticulum and the nuclear envelop of most mammals (Yang et al. 1997). Bcl-2 takes part in the complex apoptosis signaling pathway by preventing cell death without promoting cell proliferation (Chao and Korsmeyer 1998). Bcl-2 regulates apoptosis by inhibiting c-Myc or p53 activated Bax/Bid proteins, which increase mitochondrial permeability and ultimately result in cell death (Yang et al. 1997). Cancer cells often have high amounts of Bcl-2 due to an over-expressed BCL-2 gene, a proto-oncogene that can become activated into an oncogene (Gross 2001). An abundance of Bcl-2 contributes to tumor initiation, progression, metastasis, and treatment resistance (Fernandez et al. 2002, Oltersdorf et al. 2005).

Bcl-2 specific inhibitors- including drugs such as Genasense- have been developed by researchers to promote apoptosis in cancerous cells with high levels of Bcl-2 ("CancerQuest | Oncogenes: Bcl-2" 2011). In one study, a small molecule labeled YC137 was discovered and used to inhibit the anti-apoptic protein in breast cancer cells. This was the first Bcl-2 inhibitor that was able to selectively kill cancer cells that over-expressed or relied on the protein for survival, but had no effect on the majority of primary cells. Some cells did manage to develop resistance against YC137 by becoming less dependent on Bcl-2 for survival and decreasing Bcl-2 levels. But these resistant breast cancer cells did become more sensitive to chemotherapy (Real et al. 2004).

**Specific Aim 1: Selection of RNA aptamers against over-expressed Bcl-2 in cancer cells.**

Using a high affinity and specific binding RNA aptamer would be an ideal approach to studying the potentially therapeutic effects of Bcl-2 inhibitors on malignant tumor cells. Prohibiting Bcl-2’s intervention of the cell death pathway will likely stimulate cancerous cells with high concentrations of the protein to undergo cell apoptosis. A potential problem is that successful aptamers, unlike YC137, might bind to Bcl-2 in both cancerous and normal cells, encouraging apoptosis in important, primary cells. But the possibility of finding a Bcl-2 aptamer could have a positive impact on the lives of those battling cancer by aiding in mutated cell death and allowing chemotherapy, radiotherapy, and hormone treatments to be more successful (Kimball 2011, Oltersdorf et al. 2005).



Specific Aim 1

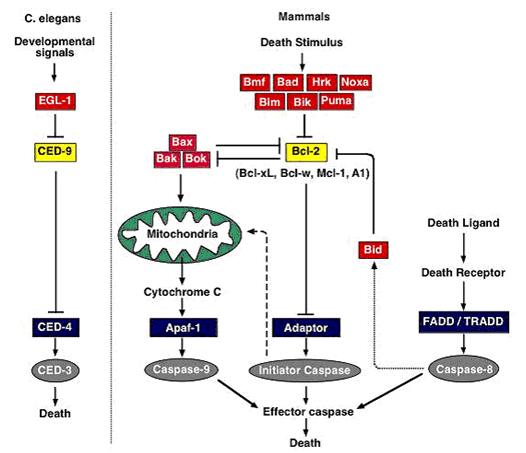
**Figure 1.** The figure above is simplified version of the p53 cell apoptosis pathway, including specific aim one. If Bcl-2 is inhibited, cancerous cells avoid survival and complete apoptosis. There are several independent signal transduction pathways (caused by different stimuli) leading to cell death that include Bcl-2. Adapted from “Nonsmall Cell Lung Cancer (2011).”

50 ug of recombinant, human Bcl-2 protein with a GST tag can be bought from the SignalChem. The catalog number is H00006531-P01 and the company can be reached at 909-839-7620. It is optimal to store the

protein in 50 mM Tris-HCl buffer at a pH of around 7.5 at -70˚C for up to a year after the shipping date.

**Introduction and Background**

B-cell lymphoma 2 (Bcl-2) is an integral membrane protein that can found in the somatic cells of most mammals. The dimer is mainly concentrated on the outer membrane of mitochondria, but can also be found in the intermembrane of endoplasmic reticulum and the nuclear envelop (Fernandez et al. 2002, Yang et al. 1997). Bcl-2 takes part in several independent, but complex apoptosis signaling pathways by preventing cell death without promoting cell proliferation (Chao and Korsmeyer 1998). This was the first protein to be associated with the inhibition of apoptosis (Fernandez et al. 2002). Bcl-2 regulates apoptosis by inhibiting c-Myc or p53 activated Bax/Bid proteins, which increase mitochondrial permeability to cytochrome c and Smac/DIABLO. These molecules signal the release of proteases through the activation of caspase cascades and ultimately result in cell protein cleavage and apoptosis (Fernandez et al. 2002, Yang et al. 1997) (**Figure 2**). Bcl-2 and Bcl-2 family protein concentrations are controlled by the BCL-2 gene.



**Figure 2.** The above figure gives another detailed look at the cell death pathway in human somatic cells. Cell death requires three types of proteins: pro-apoptotic EGL-1 proteins, CED-4 adapter proteins, and CED-3 caspases (proteases). BCL-2 and other Bcl-2 family proteins such as BCL-xL and BCL-W, are CED-9 proteins needed for cell survival. If inhibited, CED-4 proteins can bind and/or activate caspases that initiate cell death by initially cleaving proteins and fully permeabilizing the mitochondrial membrane. Organelle membranes are then made permeable, resulting in cell death. Acquired from “Research (2011).”

Abnormally high amounts of Bcl-2 and Bcl-2 family proteins, such as Bcl-x, are often found in malignant tumor cells. This is due to the over-expression of the BCL-2 gene, a proto-oncogene that can be activated into an oncogene by a translocation, resulting in its over-expression (Gross 2001). An abundance of Bcl-2 family proteins contributes to tumor initiation, development, metastasis, and treatment resistance (Fernandez et al. 2002, Oltersdorf et al. 2005). Drugs have been developed to induce cancer cell death, but are not efficient in cells that over-express Bcl-2. Bcl-2 specific inhibitors- including drugs such as Genasense, retinoic acid, paclitaxel, vincristine, and docetaxel- have been developed by researchers to promote apoptosis in cancerous cells with high Bcl-2 levels ("CancerQuest | Oncogenes: Bcl-2" 2011). The small molecule YC137 was synthesized and used to inhibit Bcl-2 in culturedbreast cancer cells (**Figure 3**). This was the first known Bcl-2 inhibitor that was able to selectively kill cancer cells that over-expressed or relied on the integral protein for survival, but had no detrimental effects on primary cells from the small intestine, lungs, and brain (Real et al. 2004). Some cancer cells did manage to develop

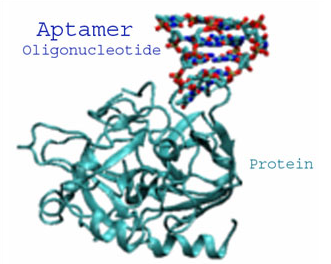
resistance against YC137 by becoming less dependent on Bcl-2 for survival and decreasing Bcl-2 levels, but these

adapted cells did become more sensitive to chemotherapy and radiation treatments (Real et al. 2004).



**Figure 3.** The figure above models the three-dimensional structure of Bcl-2 in complex with YC137. The

Bcl-2 protein is represented by the yellow, red, and purple colored ribbon model. YC137 is shown as a ball-and stick model. Acquired from Real et al. 2004.

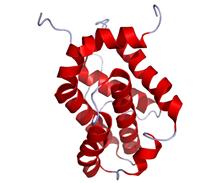
Aptamers are single stranded DNA or RNA oligonucleotides that are usually between forty and sixty base pairs long, but can be longer. These ligands bind with high affinity to specific targets, including nucleic acids, proteins, small organisms, and organic compounds (“Aptamers from Gene Link”). The binding success of aptamers can be contributed to their three dimensional folding patterns. The specific conformations these sequences take on are stable and recognize their target (**Figure 4**). The binding affinities of aptamers can be compared to, or even exceed, regular antibodies. Aptamers can have many advantages over antibodies. The cost to produce aptamers is low and they do not vary from batch to batch. Under most conditions, they are more stable than antibodies, last longer, and hardly ever provoke immune responses. These advantages make aptamers a possible or even a better alternative to antibodies in molecular diagnostics and therapeutics (Holahan et al. 2011).

**Figure 4.** The figure above illustrates the binding affinity of an aptamer, colored royal blue and red, to a specific protein (not specified on website). This image was acquired from “Aptamers from Gene Link (2011).”

Numerous Bcl-2 inhibiting molecules have been developed and the possibility of using aptamers against the protein has been discussed, but one has yet to be synthesized. A high affinity aptamer would be ideal for cancer research, having seen the therapeutic success of inhibitors against Bcl-2 in breast cancer cells and their promotion of cell apoptosis (Real et al. 2004). Aptamers could also prove useful in diagnostics, detecting cancer cells with high concentrations of proteins from the Bcl-2 family. The only potential problem is that successful aptamers, unlike YC137, might bind to Bcl-2 in both cancerous and normal cells, encouraging cell death in important, primary cells. But the possibility of finding a high affinity Bcl-2 aptamer that is able to selectively target cancer cells could have an enormous and positive impact on the lives of those battling cancer by aiding in mutated cell death and allowing

chemotherapy, radiotherapy, and hormone treatments to be more successful (Kimball 2011, Oltersdorf et al. 2005).

Bcl-2 exists as a dimer and is approximately 464 amino acids long (**Figure 5**). The average molecular weight of human Bcl-2 is 51,000 Daltons. It has an estimated pI of 7.28, suggesting that Bcl-2 has a positive charge and binds well to nucleic acids at neutral pH (1.1). As the pH becomes more basic, Bcl-2 takes on a more negative charge. The protein is found stable in Tris-HCl at a pH of about 7.5 (Putnam 2006). There are a variety of anti-cancer labs that are doing research against Bcl-2 and Bcl-2 family proteins: these include the Laboratory of Genetics and Cancer Biology at California State Polytechnic University, Pomona (headed by Dr. Steve Alas), the Alexander Lab at the University of Missouri, Abbott Laboratories (which have produced successful drugs against the Bcl-2 family), and the Apoptosis Burnham Institute laboratory. 50 ug of recombinant, human BCL-2 protein with a GST tag will undergo RNA bead based selection and can be bought from SignalChem. The catalog number is B25-30G-50 and the company can be reached at <http://www.signalchem.com> or 909-839-7620. It is optimal to store the protein at a pH of around 7.5 at -70˚C for up to a year after the shipping date.



**Figure 5.** The figure above is a three-dimensional schematic of the B-cell lymphoma 2 protein. Acquired from “Cancer Treatment (2010).”

**Experimental Design, Methods, Materials**

RNA Aptamer Selection

RNA bead based selection protocol will be followed using the R50 pool against the target. The first step of this process is immobilizing the target onto magnetic glutathione beads specific for GST chimera proteins. The beads will first be prewashed with diH2O to get rid of any impurities and then the target immobilized onto the beads after adding 1X Tris-HCl buffer at pH 7.3. While the bead/Bcl-2 mixture is incubating, a pool binding reaction will be made using 10X Tris selection buffer (200 mM Tris-HCl pH 7.3, 1370 mM NaCl, 50 mM Kcl, 20 mM CaCl2, 30 mM MgCl2) and heat denatured, forcing the RNA in the R50 pool to fold into their most stable structures. Once cooled and all unbound proteins washed away from the beads, the pool-binding reaction is added to the immobilized target and incubated at 25˚C for 25 minutes. The beads, containing the target and bound RNA, are then collected and the supernatant, full of unbound RNA, is removed and put into a tube labeled W0. The beads are then washed with three more times with 1X Tris selection buffer to remove all weakly bound aptamers. The remaining bound species are eluted (E1) with 80˚C diH2O. The RNA in tubes W0, W3 (the last wash) and E1are then ethanol precipitated. Reverse transcription will be performed on the precipitated tubes to transform the bound and unbound RNA into ssDNA. This product is then amplified using large scale PCR after the number of cycles to correctly amplify the recovered elution pool is determined by cycle course PCR, which also checks for contamination using a negative control. ssDNA will then be transcribed into RNA and PAGE purified. If the gel is successful, RNA from the gel will be eluted, precipitated, and quantified using the nanodrop program at an absorbance of 230 nm. This RNA will be used for the next round of selection.

RNA Aptamer Negative Selection

During the fourth or fifth rounds, a negative selection against the lone GST-tag and GST specific beads would have been performed as well as target selection. Remaining RNA aptamers from previous rounds would have been exposed to the tag and naked beads and diH2O used instead of Bcl-2. The remaining unbound RNA from this negative selection would have been used during the next round of normal selection, while bound RNA aptamers discarded, removing unwanted binders from the R50 pool specific to the GST-tag and beads.

The average molecular weight of human Bcl-2 is 51,000 Daltons. It has an estimated pI of 7.28, suggesting that Bcl-2 has a positive charge at neutral pH (1.1). As the pH becomes more basic, Bcl-2 takes on a more negative charge. Bcl-2 is found stable in 50 mM Tris-HCl at a pH of around 7.5. It is optimal to store this protein at -70˚C for up to a year after the shipping date. A negative selection will be performed after the third round against GST-binding RNA on the recombinant, full-length protein. 50 ug of Bcl-2 can be ordered from SignalChem at <http://www.signalchem.com> or 909-839-7620. The catalog number for the protein is B25-30G-50.

**Budget**

Bcl-2 Protein, N-terminal GST•Tag, Human, Recombinant Full-length……………….………….$395.00

50 ug

Available at SignalChem at <http://www.signalchem.com> or 909-839-7620

Catalog number: B25-30G-50

Bcl-2 is 51,000 Da (grams/mol) in weight and 50 ug costs $395. Based on this information, the cost per round should be $40.29 and there should be enough protein to complete 9.8 rounds if 100 pmol of Bcl-2 are used per round.

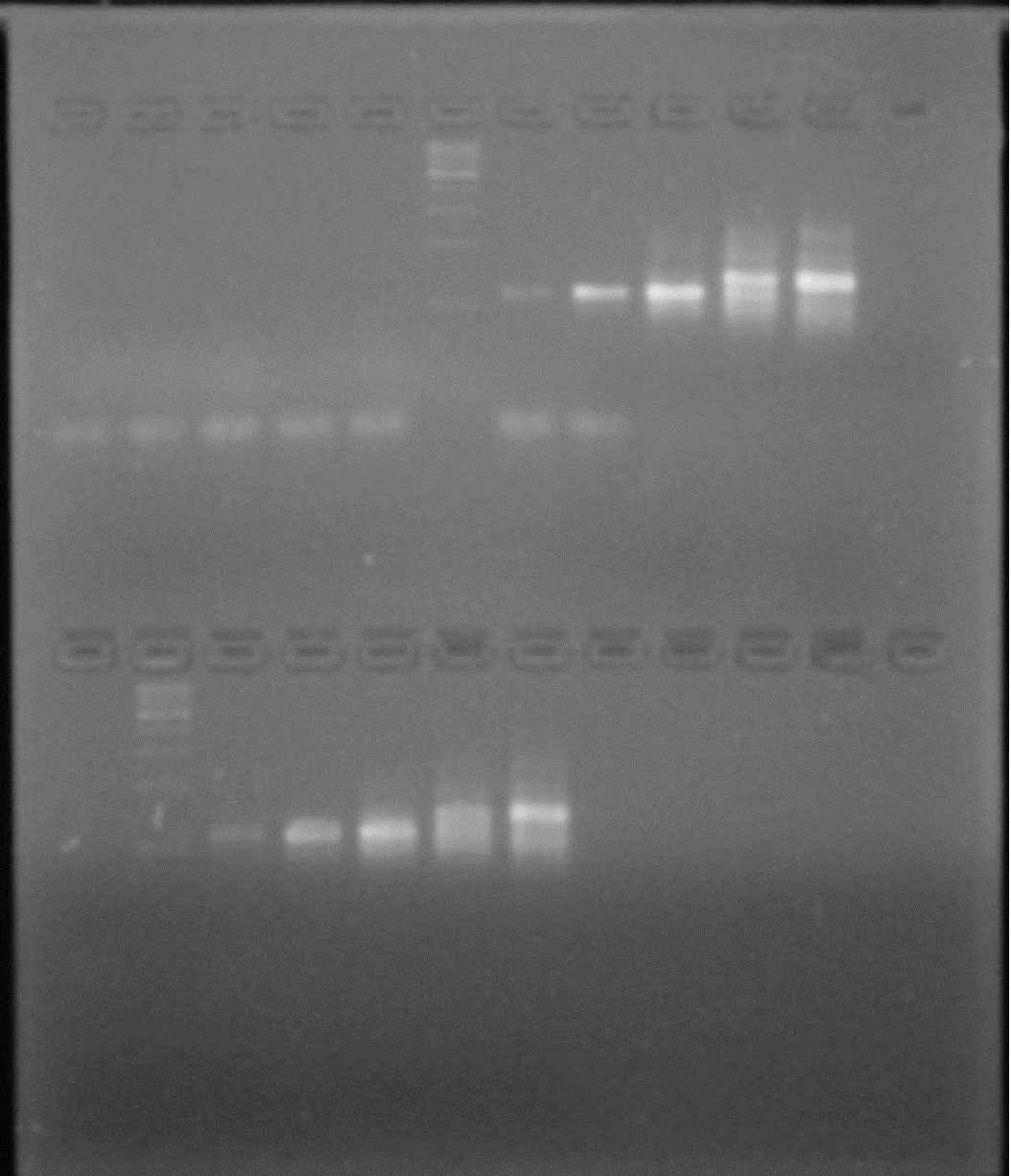
**Results and Discussion**

Bcl-2: Round 1

Complete RNA bead selection against Bcl-2 began on November 3, 2011 using the parameters in

**Table 1** (at the bottom of the section), after two failed practice rounds and a week of making Tris selection buffer components for the rest of the class. The goal of this round was to possibly produce and isolate aptamers specific to Bcl-2. This first round would not result the most specific binders, but would filter out weaker and non-binding aptamers. This round would also contain binders specific to the GST-tag and beads Bcl-2 was immobilized on, but those would have been removed if a negative selection had been performed. On November 3, the target Bcl-2 was immobilized onto beads and selected against. I completed three washes (labeled W0-W3), a mix pool binding reaction (containing RNA from the R50 pool) and an elution (labeled E1), using 2 volumes of 1X Tris selection buffer for each wash and 80˚C diH2O for E1. On November 8, ethanol precipitation and reverse transcription were performed on washes W0, W3, and E1. The reactions were stored in the freezer until November 11, when they were used for cycle course PCR. A negative control was made using all of the cycle course reaction components except for ssDNA from reverse transcription. diH2O was used instead. 5 uL aliquots from each wash were collected during cycles six, nine, twelve, fifteen, and twenty and used for gel electrophoresis. The results of the gel can be found in **Figure 6**. The wells were filled according to the following **Table 2**:

|  |  |  |  |
| --- | --- | --- | --- |
| 1. W3- Cycle 6 | 7. W0- Cycle 6 | 13. Negative | 19. E1- Cycle 20 |
| 2. W3- Cycle 9 | 8. W0- Cycle 9 | 14. Ladder | 20. Nothing |
| 3. W3- Cycle 12 | 9. W0- Cycle 12 | 15. E1- Cycle 6 | 21. Nothing |
| 4. W3- Cycle 15 | 10. W0- Cycle 15 | 16. E1- Cycle 9 | 22. Nothing |
| 5. W3- Cycle 20 | 11. W0- Cycle 20 | 17. E1- Cycle 12 | 23. Nothing |
| 6. Ladder | 12. Nothing | 18. E1- Cycle 15 | 24. Nothing |



13 14 15 16 17 18 19 20 21 22 23 24

Neg L E1-6 E-9 E-12 E-15 E-20

1 2 3 4 5 6 7 8 9 10 11 12

W3-6 3-9 3-12 3-15 3-20 L W0-6 0-9 0-12 0-15 0-20

**12 cycles for E1 during lsPCR**

**Figure 6: Bcl-2, R50, Round 1.** The first gel run after ccPCR in Round 1 is depicted above and appears to have been successful. W0, the positive control, showed up and amplified around the same time as E1 (after 12 cycles), but appears to have over-amplified more than E1. This over-amplification was expected because W0 contains all the RNA strands that did not bind to Bcl-2. Also, E1, which contained potential aptamers against Bcl-2, showed bands before the last wash (W3), suggesting that all possible binders were collected and all weak or nonbinding proteins were removed by the last wash. A well defined band for E1 (before over-amplification) occurred at cycle 12. E1 for the twelfth cycle is boxed in red above. This defined band determines the number of cycles that will be used during lsPCR.

Large scale PCR was then performed on November 11 to make enough E1 template for transcription. Six

100 uL reactions were made, each containing E1 from reverse transcription. These six reactions were run for twelve cycles as determined by gel electrophoresis after cycle course PCR. On November 15, the reactions were combined, ethanol precipitated, and transcribed. On November 16, a PAGE gel was run. The PAGE gel appeared to be successful and is depicted in **Figure 7**. A portion of the gel was incubated at 37˚C overnight, and the eluted RNA was prepared for transcription the next morning. Ethanol precipitation was continued on the gel elution on November 21 and quantized, producing a nanodrop value of 938.6 ng/uL. This is equivalent to an RNA aptamer concentration of 28.05 pmol/uL (uM). 7.13 uL of purified binders from this round is equivalent to 200 pmol that will be used next round. These aptamers will be selected against again so that all low and medium affinity binders are removed. There was not enough time to perform a negative selection round, so it is uncertain what percent of

binders from the end of round one are GST bead and tag specific.

Portion cut out; amplified RNA binders from R50 pool seen under UV light

Seen with naked eye

1 2 3- E 4 5 6- JB 7 8

**Figure 7: PAGE Gel Results for Round 1, Bcl-2, R50 pool.** The PAGE gel for the end of round one appears to be successful, although the amplified RNA cut out was lumpy and not in one compact shape. The RNA cut-out was placed in 3 mL of 1X TE and incubated overnight so that the purified RNA binders could be eluted from the gel. I ran my reaction with Elena.

|  |  |
| --- | --- |
| Initial Conditions and Parameters for Bcl-2 | |
| Target | Bcl-2 |
| Beads | Glutathione beads specific for GST chimera proteins |
| Pool | R50 |
| Incubation time/temperature | 25˚C for 25 minutes |
| Buffer and pH | Tris-HCl buffer, pH7.3 |
| Salt and Ion Concentration | 200 mM Tris-HCl pH 7.3, 1370 mM NaCl, 50 mM Kcl, 20 mM CaCl2, 30 mM MgCl2 |
| Wash Volume and Number | 3 washes, 2 volumes |

**Table 1: Initial Conditions and Parameters for Bcl-2 Using R50 pool.**

Bcl-2: Round 2

The second round against Bcl-2, also using the complete RNA bead selection protocol, began on November 21. The main objective of this second practice round was to continue selection and narrow down the pool of potential aptamers against Bcl-2. Target immobilization was performed using Tris buffers I had made earlier in the month. A mix pool binding reaction was made using 200 pmol of strong binders from the previous round. Three washes (labeled W0-W3) and an elution (labeled E2) were completed for this round, using 2 volumes of 1X Tris selection buffer for each wash and 80˚C diH2O for E1. The reactions were then stored in the -20˚C freezer.

The goal of completing two to three practice rounds was not completed and a negative selection was not conducted. But I have mastered the RNA bead based selection protocol. In the previous practice rounds with lysozyme, having had no previous experience with the protocol, I made a few small, but crucial mistakes that compromised my rounds. I was able to perfect my techniques, such as making a negative control during cycle course PCR, and thoroughly following the protocol. As of now, it can be concluded that most of the strong and medium affinity binders for Bcl-2 have been isolated, but there is a proportion of those binders that are GST bead

and tag specific because a negative selection round has not been completed.

**Overall Problems Encountered**

Bcl-2 Round 1

No errors were made during the first round of selection. The protocol was followed thoroughly, negative controls made for cycle course PCR, both gel electrophoresis and PAGE appeared successful, and an adequate amount of RNA aptamers from the R50 pool were amplified and purified (as determined by the nanodrop calculation). Another gel after large scale PCR could have been run to double-check for contamination and the

correct amount of amplification (determined by the clarity of the gel electrophoresis band and having no over-amplification). Gel electrophoresis would have been performed after large scale PCR in round two.

Bcl-2: Round 2

No mistakes were made during round two. Only complete target isolation, binding and selection against the remaining aptamers from round one, and elution were performed for this round.

**Conclusion and Future Work**

The objectives of these first few rounds of RNA bead based selection were to find aptamers specific to Bcl-2, select against weaker binders, and to get rid of stronger binders that were GST bead and tag specific through negative selection. The latter was never determined, however, because a negative selection was not performed. The first round was successful, producing a gel without contamination after cycle course PCR and a PAGE gel with an adequate amount of purified, eluted RNA (938.6 ng/uL). It cannot be determined yet if round two is successful because cycle course PCR and gel electrophoresis have not been performed. A summary of the first two rounds of selection against Bcl-2 can be seen below in **Table 3**.

|  |  |  |
| --- | --- | --- |
| RNA Bead Based Selection Against Target Bcl-2 Using Pool R50 | | |
|  | Round 1 | Round 2 |
| Cycle Course | Cycle course was successful; no contamination seen by the negative control, W0 over-amplified before E1, and no amplification for W3. It was determined 12 cycles were needed for large scale PCR. | n/a |
| Absorbance | 230 nm | n/a |
| Quantity of Nucleic Acid Used | 200 pmol provided from R50 aliquot | 200 pmol (7.13 uL from the end of round one) |
| Quantity of Nucleic Acid Recovered | 938.6 ng/uL suspended in 30 uL 1X Tris Selection Buffer (equivalent to 28.05 pmol/uL or uM) | n/a |

**Table 3: Summary of RNA Bead Based Selection on Bcl-2 Using the R50 Pool by Jessica Beardsley.**

If selection on this project were continued, a negative selection against the GST specific beads and the GST-tag would have been completed alongside round four or five. This would have been completed by skipping the target immobilization step so that all aptamers specific for the beads and not Bcl-2 could be removed and possibly identified. After three or four more rounds, a binding assay would have been performed to see the exact sequence of binders against Bcl-2.

**Student Story**

My plans for the future have not changed because of the FRI program. I came in wanting to be a doctor or a physician’s assistant, but know if those two options do not work out I would be more than willing to pursue a career in research. Bench work can become tedious at times, but research as a whole is exciting. The thought of discovering something that can change the world is exhilarating. But with discovery comes trial and error, I have realized. Many mistakes are made, but the FRI has taught me patience and perseverance. When errors are made, I am forced to think critically about what I have done wrong, but my experience in the FRI has made me confident in my problem solving skills, enough so that I am now willing to discuss my ideas with my peers.

I have a sense of pride knowing that most people do not get the opportunity to do what I am doing now until they enter graduate school. The FRI program has opened the door to many opportunities, allowing me to push myself further intellectually than I could have imagined. My understanding of science has increased exponentially since high school and I enjoy sharing my knowledge I have gained from the FRI and the College of Natural Sciences. The research I am doing can seem overwhelming to those who study outside of the sciences, but I feel that I am able to explain my work relatively well and in terms that are simple enough for everyone to understand, but are not oversimplified. The FRI program has made me a better science student by allowing me to better understand the material I am learning in the classroom through my work in the lab.

I cannot think of any changes I would make to the FRI program. I have enjoyed my experience, have been given many opportunities to continue research, and learned more than I could have ever imagined. As for the Aptamer stream, the only minor change I might make is how the lab hours are handled and graded. At first, I was not sure if I could handle ten lab hours every week with a part time job, but adjusted and was able to keep up with them throughout the semester. A great portion of the students, however, seemed to have struggled with the lab hour requirements and have had to make up as many as thirty hours the last two weeks of school. Maybe instead of waiting until the very end of the semester to grade lab hours, there should be checkpoints around midterms in which a certain amount of hours should be completed and graded. It might give students more of an incentive to come into lab earlier and keep up with the hours.

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