Selection of RNA Aptamers that Inhibit Mutant 3 HIV-1 Reverse Transcriptase

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Table of Contents

Acknowledgements

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Abstract

Acquired immunodeficiency syndrome (AIDS) is one of the most widespread diseases on the planet, and the need for new forms of treatment has become paramount. Even though there are treatments against human immunodeficiency virus (HIV), the virus mutates readily and becomes resistant to these drugs. A mutation is a slight change in the virus that allows it to evade the action of the medication. Because resistance to anti-viral drugs is pervasive, it is imperative to find new ways to treat such mutant viruses.

A promising field for the treatment of HIV is the application of aptamers. Aptamers are nucleic acids that form tertiary structures, which can bind to proteins tightly and selectively. If an aptamer bound and inhibited a key enzyme in the virus, it could help prevent the spread of HIV in the human body. An important target for therapeutics has been reverse transcriptase, which is vital for viral reproduction. Although aptamers that inhibit reverse transcriptase (RT) activity have already been isolated, these are against wild type HIV, or most common form of the virus. Thus, it is important to select for alternative aptamers that will inhibit other drug resistant forms of the virus.

The Stanford University HIV drug resistant database lists twelve common drug resistant HIV variants with mutant forms of RT. The object of this study was to select a few aptamers that inhibited the activity of mutant clone 3 RT from the aforementioned database. Aptamers were found not only to prevent enzymatic action of RT, but also bound to other regions of the protein itself.

This led to the testing of innovative techniques within the realm of aptamers by creating complexes that link two aptamers together, which can bind more tightly to the enzyme because there are more contact points between the aptamers and protein target.

These findings also open the door to improving current aptamer microarray assay technology. Microarrays utilize aptamers that are placed on slides to detect the presence of a certain protein. The improvement comes from using aptamers, instead of antibodies (another molecule that binds to proteins) as the signal molecule for the test. Thus, aptamers can be used for both therapeutic and detection means against HIV.

Introduction

Acquired immunodeficiency syndrome (AIDS) is one of the most widespread diseases on the planet, and the need for new forms of treatment has become paramount. Current treatments focus on various mechanisms of the human immunodeficiency virus type 1 (HIV-1), including replication, integration and proliferation of the virus. The development of highly active antiretroviral therapy (HAART) has provided a promising means to treat patients infected with HIV-1. HAART utilizes a combination of medications to increase the efficacy of treatment, as well as, decrease the prevalence of drug-resistant viral strains. Almost half the drugs of HAART work to inhibit the activity of reverse transcriptase (RT), which is the enzyme that is needed to convert the RNA genome of HIV-1 to DNA so that it can become integrated with the human genome (1). Anti-RT drugs are divided into two categories: nucleoside reverse transcriptase inhibitors (NRTI's) and non-nucleoside reverse transcriptase inhibitors (NNRTI's). NRTI's are structurally similar to the monomers of DNA, except that the drugs lack a 3'-hydroxyl group and function as chain terminators. The NRTI's are taken up by the cell and must use the cellular kinases to become phosphorylated, which makes the drug active. RT then incorporates these nucleoside analogs when converting the virus's genome into DNA and leads to truncated products (2). NNRTI's work by binding to a hydrophobic pocket on RT that is near the active site for polymerization. The exact mechanism for inhibition is not known, but studies suggest that DNA catalysis step is hindered (3, 4).

Even though these anti-HIV treatments have been proven effective, the virus mutates readily and becomes resistant to these drugs. HIV-1 mutates at a high rate due to the low fidelity of RT, which incorporates mutations at a rate of 3.4×10^{-5} per base per

cycle of replication (5). This translates to approximately one mutation for every 1-2 copies of the virus (6). Due to the high prevalence of mutation, HAART ceases to work in many HIV-1 patients (7). The Stanford University HIV drug resistant database now lists twelve common drug resistant mutant variants of RT, as described by Johnston and coworkers (8). Because resistance to anti-viral drugs is pervasive, it is imperative to find new ways to treat such mutant viruses. A medication that is able to maintain its effectiveness even when its target is constantly changing is desirable. Conversely, developing more inexpensive diagnostic assays that can differentiate between various drug resistant variants of RT also remains a goal.

Current anti-HIV therapies utilize a "guess and check" method to see if a certain cocktail of drugs proves to be effective for a given patient. However, due to the side effects of many of these medications, it would be much more beneficial to accurately treat the specific virus for each individual patient. Current diagnostic techniques to look for mutations are fairly expensive and difficult to perform in a global manner. Most of these assays are used more for academic use, rather than medical use. Two examples of commercial phenotypic assays are Antivirogram and PhenoSense. These assays work by isolating and amplifying the RT gene, and then inserting the gene into a viral genome, which is used to infect cell lines. Viral growth is then measured in the presence of different anti-viral drugs to determine resistance (9). Genotypic assays, such as Trugene, rely on direct sequencing of the viral genome in order to determine which HIV-1 mutants a patient is infected with. These assays require various amplification and electrophoresis steps that are restricted to laboratories and can cost about $500 per assay (10,11). So even though there are methods to test for drug resistant strains in patients, most HIV-

infected individuals are unable to access this technology. Consequently, an assay that is less expensive and employs a simpler system would have great utility.

Aptamers have developed as a technology that can function as a solution to both the diagnostic and therapeutic concerns stated above. Aptamers are nucleic acid oligonucleotides selected to bind molecules with a high affinity and specificity (12,13). The technology is very recent and based on the method of *in vitro* selection, which evolves ligands against a target from a synthetically produced nucleic acid library (14,15).

A therapeutic application is to use aptamers to inhibit protein activity by either binding to the active site (in the case of an enzyme) or prevent its interaction with other proteins. Currently, there is only one drug in the market that utilizes aptamers. This drug, called Macugen, acts by binding to a vascular endothelial growth factor (VEGF) in the eye in order to treat age-related macular degeneration (16-18). However, there are other aptamer treatments that are currently undergoing trials. Specifically there are two drugs that function as anticoagulant agents that have completed Phase I trials (19-20). Aptamers have the additional advantage due to their target specificity, which leads to fewer non-specific effects within the body because they are less likely to bind other proteins. For instance, two aptamers that differ by 3 out of 44 nucleotides can differentiate between citrulline and arginine, which are very similar molecules (21-22). Another benefit is that the pharmacokinetics of aptamers can be controlled by using an anti-sense oligonucleotide to disrupt the aptamer structure, which prevents the drug from binding to its target (20). *As a result, if there are side effects from the aptamer drug, the effects can be quickly neutralized.*

RNA and DNA aptamers have been selected to target and inhibit wild type HIV-1 reverse transcriptase (RT), and consequently can be utilized as a therapeutic tool (23). These aptamers bind to WT HIV-1 RT in the low nanomolar range and form a tertiary structure that binds to the primer-template cleft in the enzyme (24-25). The first class of aptamers selected against WT HIV-1 RT was a set of RNAs containing a psuedoknot structure (26). Since then, other RNA aptamers have been isolated and contain either a stem loop or psuedoknot structure (27-28). DNA aptamers have also isolated and contained pseudoknots, hairpin loops and G-quartet motifs (29-30). These aptamers have also shown inhibition of RT activity *in vivo* through cell cultures studies (31-32).

However, almost all of the selected aptamers target only WT HIV-1 RT, and these aptamers have not been well characterized against drug resistant variants of RT. A recent study looked at mutant 3 HIV-1 RT from a panel of 12 multidrug-resistant HIV-1 RT variants (8, 33). It was found that the canonical RNA psuedoknot aptamer did not bind to the mutant variant. Also, two aptamers were isolated where one could discriminate and one could not discriminate between the two types of RT. However, both aptamers did not inhibit enzyme activity (33).

The target protein for the selection was the mutant 3 HIV-1 RT. Mutant 3 HIV-1 RT is highly resistant to 3TC (Lamivudine) (>300 fold decrease in effectiveness) and AZT (Zidovudine) (60 fold decrease in effectiveness) and is somewhat resistant to ABC (Abacavir), ddI (Didanosine), d4T (Stavudine) and TDF (Tenofovir) (34). This mutant HIV-1 RT contains 10 different residues from the wild type enzyme - T39A, M41L, K43A, E44D, D67N, T69, V118I, M184V, L210W and T215Y. Four of these mutations (M41L, D67N, L210W and T215Y) are in the family of thymidine analog mutations

(TAM's) and impart a resistance to thymidine analogs, such as AZT (35-36). The presence of multiple TAM's, such as the M41L, L210W and T215Y triad that is seen in 4 of the 12 mutants, also causes general resistance to NRTI's (8, 37). When the E44D and V118I mutations occur in the presence of other TAM's, a dual resistance against AZT and 3TC is conferred upon the enzyme due to the increased excision of incorporated 3TC-monophosphates (38-39). Aptamers that target the mutant 3 variant may be able to distinguish it from other drug resistant mutants from the panel. Conversely, due to the similarities among the different mutants, an aptamer that binds to mutant 3 may be able to bind other drug resistant variants with similar mutations. For instance, binding studies were done between mutant forms of nonstructural protein 3 (NS3) of Hepatitis C virus (HCV) and anti-NS3 aptamers, and it was found that some mutants bound with similar affinity to wild type protein, while others did not bind at all (40). Also, DNA aptamers have been isolated that are relatively promiscuous and can bind to various forms of reverse transcriptase, including HIV-1, HIV-2 and SIV_{CPZ} (41). Consequently, from a single selection, it may be possible to isolate aptamers that fall into each of these two categories.

Also, a previous selection against mutant 3 RT found aptamers that bound to unique epitopes distant from the active site of the enzyme (33). It is plausible that this selection will also yield non-inhibiting aptamers that bind to RT with a high affinity. A set of aptamers that bind to separate regions of a protein can be linked together to create a moiety that is able to bind more tightly to the target than any of the individual aptamers. For instance, two anti-thrombin aptamers that bound to different epitopes of the protein were attached using phosphoramidites linkers, and the resultant molecule bound 9-fold

greater to thrombin than a single aptamer alone (42). Thus, it may be possible to create a system of aptamers that bind to separate epitopes on RT that is able to bind with a higher affinity using multivalent interactions.

Methods and Materials

In vitro Selection

Mutant 3 HIV-1 RT was prepared using the protocol from Li et al. The RNA pool was transcribed from a dsDNA library that consisted of a 100 nucleotide random region and is surrounded by constant regions where a forward and reverse primer can sit for amplification. The general DNA sequence of these aptamers is as follows: TTCTAATACGACTCACTATAGGACAGCAAAGCGTACATCTA – N100 – TGCTAGCCTGAAGTCATACG (the T7 RNA promoter is underlined). With a pool complexity of $10^{13} – 10^{14}$ sequences, approximately 3 copies of each sequence was transcribed from the dsDNA library. The RNA pool was treated with DNase to remove the DNA templates and then gel-purified and concentrated using ethanol precipitation. For the binding reaction, 200 pmol of pool RNA was incubated in 1X Selection Buffer (20mM HEPES pH 7.5, 5mM $MgCl_2$, and 150mM NaCl) and denatured at 70˚C for 3 minutes and then cooled to 25˚C. Then 50 pmol of Mutant 3 HIV-1 RT was added to the reaction (200uL total reaction) and incubated for 30 minutes at 25˚C. The binding reaction was then passed through a nitrocellulose filter (0.45µm HAWP disks from Millipore, Bedford, MA), which was prewetted with 100uL 1X selection buffer, and washed with 4 volumes of 200uL 1X selection buffer The RNA captured on the filter was eluted in 400uL elution buffer (7M Urea and 25mM EDTA) at 100˚C for 5 minutes.

The eluant was then phenol-chloroform extracted to remove target protein, and the remaining aqueous solution was ethanol precipitated to isolate and concentrate remaining RNA. RNA was amplified through a process of reverse transcription, PCR and *in vitro* transcription to create the pool for the next round of selection. Starting with the third round of selection, a negative selection was conducted where prior to adding protein in the binding reaction, the reaction is passed through a nitrocellulose filter and liquid that passes through the filter is used for the binding reaction. After 13 rounds of selection, the DNA template for that RNA pool was cloned into a TA Vector (Invitrogen), transformed into TOP10 Competent cells and plated. Forty colonies were sequenced, and 20 unique aptamers were found.

Filter Binding Assays

Single Point Binding Assay

Relative binding of aptamers was ascertained by first labeling individual aptamers radioactively (internal α-^{32}P). The aptamers were first denatured for 3 minutes at 70˚C and cooled to 25˚C. For the binding reaction (50uL reaction done in triplicates), each aptamer was incubated in 1X selection buffer with target protein in a 1:1 aptamer:protein ratio at 100nM concentration for 30 minutes at 25˚C. The reaction was pushed through a nitrocellulose filter and then a nylon filter on a vacuum manifold. The nitrocellulose filter captured aptamer-protein complexes, and the nylon filter captured the remaining aptamers not bound to protein. A Phosphorimager was used to quantitate and percent binding was determined by counts on nitrocellulose filter / total counts on both filters x 100. These assays were conducted against both Mutant 3 and Wild Type HIV-1 RT.

K_d Assay

The dissociation constants for the top six binding aptamers against Mutant 3 HIV-1 RT were ascertained using a filter binding assay where the protein concentration was varied from 0.1nM to 1000nM, depending on the individual aptamer. In the binding reaction 0.25pM end-labeled aptamer (γ-^{32}P) was incubated with 8 different concentrations of HIV-1 RT in 1X Selection Buffer for 30 minutes at 25˚C. Each reaction was then sieved through the same set of filters as in the single-point binding assay scheme and quantitated using a Phosphorimager. The data points were then fit into a sigmoidal curve, and the Kd was determined to be the concentration at which half the ligands were bound. The dissociation constants of all six aptamers were determined for both Wild Type and Mutant 3 HIV-1 RT. Additionally, for aptamer C3, the dissocation constant against Mutant 5 and Mutant 9 HIV-1 RT was determined in the same manner

Competition Binding Assay

Competition of TPK 1.1, C1, C3, C5, C13, C16 and C24 was determined by performing assays in a combinatorial manner. First, seven binding reactions of 10nM TPK 1.1 (internal α-^{32}P labeled) were added to 50nM of Wild Type HIV-1 RT and 500nM of each individual unlabeled aptamer (one per reaction) in 1X Selection Buffer. There was also a negative control where no unlabeled aptamer was added. These sets of binding reactions were repeated for each individual radiolabeled aptamer. For C16, the assay was repeated using an altered protocol. These binding reactions were altered such that 25pM C16 (end γ -^{32}P labeled) was used instead of 10nM of internal α-^{32}P labeled aptamer. The binding

reactions were incubated for 30 minutes at 25˚C. Each reaction was then sieved through the same set of filters as in the single-point binding assay scheme and quantitated using a Phosphorimager. Percent binding was determined by counts on nitrocellulose filter / total counts on both filters x 100. Two aptamers would be deemed to compete for the same site on HIV-1 RT if the percent binding decreased when unlabeled aptamer was added with reference to the negative control.

Filter Binding Assay with Varying Aptamer Concentration

25pM end γ-^{32}P labeled C16 RNA was combined with unlabeled C16 RNA to final concentrations of .025, .1, .25, 1, 2.5, 10, 25 and 100nM C16 RNA. The RNA was denatured for 3 minutes at 70˚C and cooled to 25˚C. For the binding reaction (50uL reaction done in triplicates), each aptamer was incubated in 1X selection buffer and 200nM Mutant 3 HIV-1 RT for 30 minutes at 25˚C. Each reaction was then sieved through the same set of filters as in the single-point binding assay scheme and quantitated using a Phosphorimager. Percent binding was determined by counts on nitrocellulose filter / total counts on both filters x 100.

Inhibition Assays

Polymerase Inhibition Assay

Sequences for assay primers and templates from Li et al. A stock template:primer mix (5X) was prepared by denaturing 25nM reverse primer (end γ -^{32}P labeled) and 125nM RNA template in 1X Selection Buffer at 70˚C for 3min and slowly cooled by 1˚C/s increments to 25˚C. The polymerase activity of HIV-1 RTs were assayed by incubating a

1X template:primer mix with 50 mM of dNTPs and 50 nM RT in Selection Buffer at 37˚C for 10 min. Different concentrations of aptamers (ranging from 1 nM to 1000 nM) were added to the reactions. Reactions were quenched by adding 2X denaturing dye (7M Urea and 0.025% bromophenol blue) and heated to 85˚C for 5 minutes. cDNAs were separated from unextended reverse primers by 6% PAGE and quantitated using a Phosphorimager.

RNase H Inhibition Assay

Sequences for assay primers and templates are same as in polymerase inhibition assay. A stock template:primer mix (5X) was prepared by denaturing 25nM RNA template (end γ -^{32}P labeled) and 125nM reverse primer in 1X Selection Buffer at 70˚C for 3min and slowly cooled by 1˚C/s increments to 25˚C. The RNase activity of HIV-1 RTs were assayed by incubating a 1X template:primer mix with 50 nM RT in Selection Buffer at 37˚C for 10 min. Different concentrations of aptamers (ranging from 1 nM to 1000 nM) were added to the reactions. Reactions were quenched by adding 2X denaturing dye (7M Urea and 0.025% bromophenol blue) and heated to 85˚C for 5 minutes. cDNAs were separated from unextended reverse primers by 8% PAGE and quantitated using a Phosphorimager.

Native Gel Electrophoresis

Native gel electrophoresis was used to look for homodimer formation of aptamer. Varying amounts of unlabeled C3 was added to 1nM of end γ-^{32}P labeled C3 in 1X Selection Buffer such that the final aptamer concentration was 1, 10, 25, 100, 250 and

1000nM (final volume was 8uL). This process was repeated for C16. Each reaction was denatured at 70˚C for 3 minutes and then cooled to 25˚C. 2uL of 60% glycerol was added to each reaction in order to ease loading of sample into well. The samples were separated on 8% non-denaturing PAGE (no urea in gel) at 4˚C and visualized using a Phosphorimager.

Aptamer Duplex Formation

The TPK 1.1 sequence was obtained from Tuerk et al. 1992, and the sequence was as follows: GGGAGCAUCAGACUUUUAAUCUGACAAUCAAGAAUUCCGUUUUCA GUCGGGAAAAACUGAACAAUCUAUGAAAGAAUUUUAUAUCUCUAUUGAAA C (26). 15 nucleotide linker regions were added to the 3' end of Clone 16 and the 5' end of TPK 1.1. The linker sequence is 45 nucleotides long with a 15 thymidine region flanked by 15 nucleotide regions that hybridize to the linker regions of each aptamer. The linker is also modified to which the 3'-OH end of the oligonucleotide contains an additional alkyl spacer to prevent further extension via a polymerase. The linker sequence is CGTGACATGGATCCCTTTTTTTTTTTTTTGTATATCGATCGTAT, where the 5'end of linker binds to TPK 1.1 and 3' end of linker binds to Clone 16. The duplex is formed by denaturing the two aptamers and linker sequence at 70ºC and allowing the oligonucleotides to anneal and reform their native states. The concentration of each part of the duplex is varied dependent on the assay.

Results and Discussion

In vitro selection of aptamers that bind to drug-resistant mutant 3 variant of HIV-1 RT

The initial pool for the selection consisted of an RNA library with a length of 140 nucleotides, which contained a 100-base random region flanked by two 20-base constant

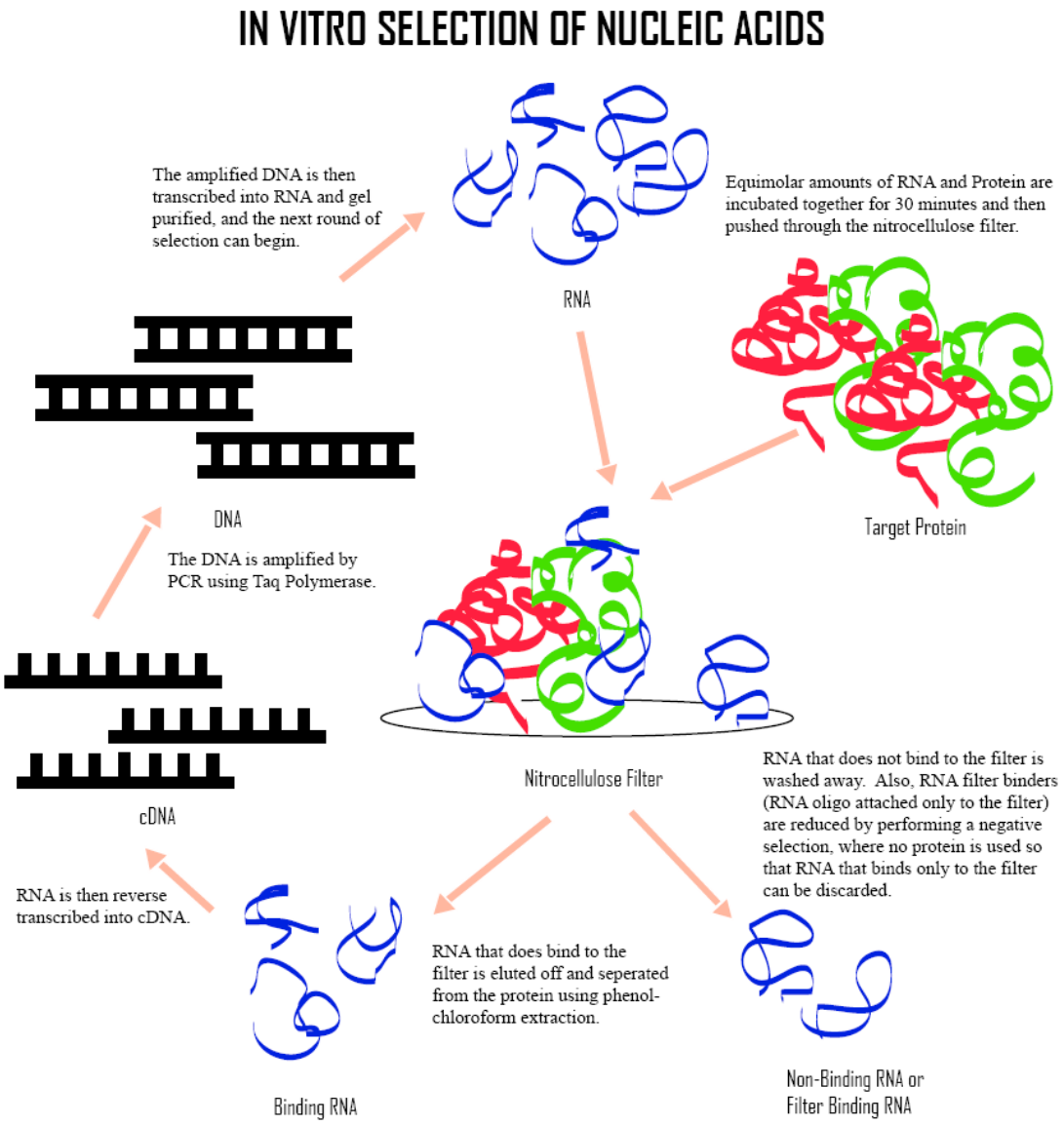

Figure 1 – Diagram demonstrating the steps for *in vitro* selection against a protein target utilizing a nitrocellulose filter to partition between binding and non-binding species.

regions. The selection against mutant 3 HIV-1 RT was carried out for 13 rounds using a nitrocellulose filter to partition the RNA bound to protein from the free RNA. The scheme for a round of selection is shown in figure 1.

The progress of the selection was followed by performing single point binding assays after certain rounds to determine the amount of RNA from the pool that bound to the target protein when incubated in a 1:1 ratio. The percent binding increased from 8% from the starting pool to 30% after round 13. The overall enrichment of the pool after certain rounds can be seen in figure 2. The pool looks to have reached its maximum binding potential by round 11 and is clearly saturated by round 13. A negative selection was also performed every round starting with round 3 in order to prevent the evolution of aptamers that bind solely to the nitrocellulose filter. The success of the negative selection is seen in the <1% binding for the no protein samples in the single point binding assays.

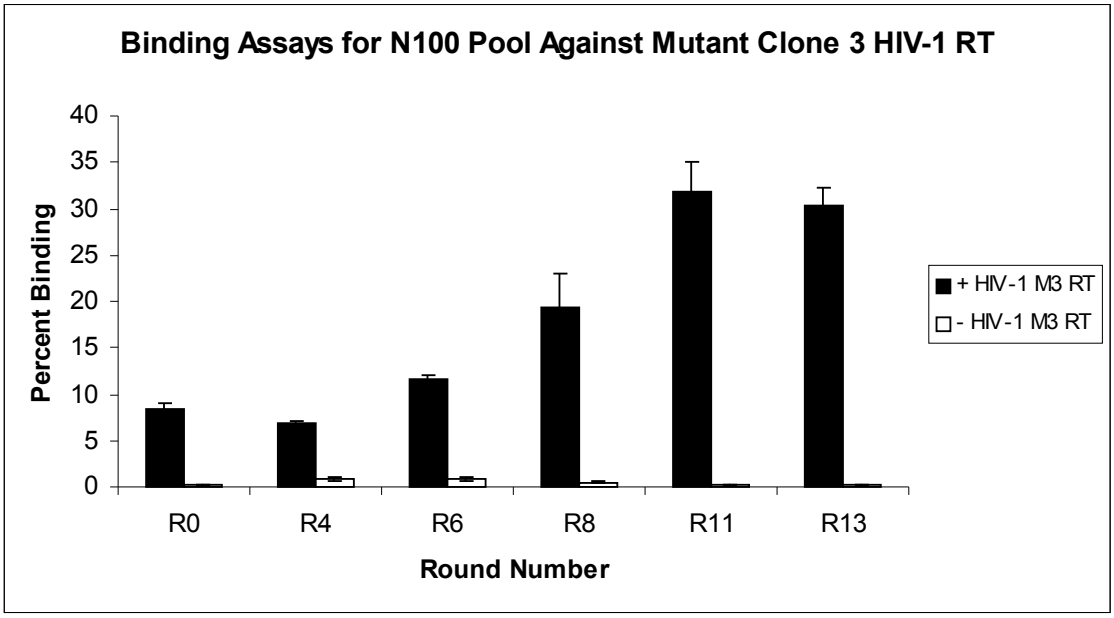

Figure 2 - Single point nitrocellulose binding assay for the N100 pool after 13 rounds of selection against mutant clone 3 HIV-1 RT. Protein and radioactively labeled RNA are 1:1 ratio at 250nM.

After 13 rounds of selection, the pool was cloned into a vector, and 30 aptamers were isolated and sequenced. Among the sequenced aptamers, there were only 20 unique clones. The sequences of the clones are represented in figure 3. Overall the pool contained a variety of different sequences, where only Clone 5 had more than two

>**Clone 1** (2) – UGCUGACGGCGUGAUAUAAUAAUAACCUACAUGCUCUUCGGCCUUCGGAAGAAU
CACGCUAGAGUAGCGAGUCAGCGAACACGCAUCUAUGACCAGGUUA
>**Clone 2** – CUGCCAUAAUAUUUCAAACUUUCUAAUCAUUUUCCUGUUCUUAUUCUGCCGGAAUCU
UUUCACGCAUUUUCUCUUAUUCCGGCUCCUG
>**Clone 3** – UGAUGCUGUAAUUGGCAUCCAAUCUGCGCCAACAGCCUUUUUAGUCGUUACUUGAG
UAGCGUGCGAUUUGUGAAACCGCUCAAUACGACUAGGUAGAG
>**Clone 4** – ACCUACUUAAAUUAAAUUUCUACUCGUUCCUCUUUUUUUUUUUUUCUUUACCGUCGUUU
CCAUUUUUAAUUAGACACUCUUUAUCAACAUUCACUCG
>**Clone 5** (4) – AAUGUAUAAGGAGAGCUCCGUAAGGACUGUCCCGCCUAUGACUGUCGACUCAG
GUCGUAAAACUGCCUCUCAGGAUACAAGUAUCACCUAGUGUUGGCA
>**Clone 7** – UAUUAUCCGGUGCUCCCGCCACUAAGUUAAAUGCUUGCGAAAUACUAAUAACAAUA
AAGUCCAUCCUUUCUUACCCAUACUCCAGAUUCCCGACUAAGC
>**Clone 9** (2) – ACCCCGACUCGUGUUAGCACAUAGGACGGGUGCUGACCCCCUGCAACUAACUCC
UCAAGGGAUACAAGCGCGGAGUCUGGCACUAACCAAGUACCGGG
>**Clone 11** (2) –CCUGCGCUAAGAAAAGAUCUAACUUGAUUUGUCAAGGAAUACACCGCAGGGUA
UUCACCCUUAAAAAUAUCGACAUCCAUCUAGCAUCCACGAUCACCCG
>**Clone 13** – GUCUGAGGAACCAGUACCUCCGCCGCAGGUAGUCCACCUGUUAAGUAAGAUCCUA
CGACUUGACCCUCGCGUCUCUUAUUAUCGAGACCCGGCUUCAUGA
>**Clone 14** (2) – ACUGGACGGAUUACAAAGUGCGACGCCAAUUCUCAAGGUCACUAACGUUUCAC
CUUCGGGUCGUAAAACCAGCUUGUAUACACGAUCGUUUCCCAAGUUG
>**Clone 16** – GUCGCUAACAAACUCAUUCUUAUAACACGUCUAUCUACUCCUUUUUAGUAAUUCAA
CUUGAAUGCUUAUCUUUUUUUUCUUAUACUCCCGCUUUG
>**Clone 20** – AUUUUCGUUGAUGCGGAUACCUGAACCAUGGACUUGGUCGACCUCAAUGUAAAAC
AGGUGAAUCGCUAACUUAGGAUUGCACCACGCUAUGAGAGGUCGUC
>**Clone 24** – UGCCGCAGCCUCUAAGUGUAAGCAUCAAUCUGGGUAAACCCGUAGACGAAAUAUG
GCUCUCGAUCCGUUAGGGAGGCAAGACUACGGACCCAGUUUUGCG
>**Clone 27** – AUCCCUCACUAGCGCUAUGGCCCGGCAAUUAAUCUCCGUUUCAAACCCCACUAUCG
AUUCACAAUCGGACAAUAUUGUGACACCUUCACCUAUA
>**Clone 28** – AGGGCCCCUAAUAAUAAUAAUCCUGUGACAAAGAUCACGAUGGGGCCCGCUAUAG
AUAUCGUUGUUCAACAAUAAUCCCAUGUCAUCUUCUGCCCUCGUG
>**Clone 30** – AACACAGGGUGGCUAACCCCUCAAAGUAGACUGUUAAGGACUUCACGGUCGUAAA
ACCAACUCCACGGGUGUCUCCUGAUGGCGUUG
>**Clone 31** – GCAAUUUAAGGCAUGAAAAUCGUACACAUAACAUGGCCUAUCUACGACCGUACGU
CAAUCACUGUCAACCCACAACUGUGGUUACUUGGUUUCC
>**Clone 34** - GUCCAAUGUACCUUACGCAGUGACAGUUACUAAGCGUGCGUCACUGACGCUUGACC
CAAAAUUUAAUAUUGACCGAUGGACGCUACCUGA
>**Clone 35** – AUAACAAUAAUAAUAUUCGUACAGUGCUGCCGCCUUUGAAGAUAACCGCUCACUU
GUAAGCGGGGAAGUUCGGCUACGGGGUUAGCAAGGUGCGG
>**Clone 39** – CUAGCCUACGCUUUCUAUUAUAACAUAAACAUAUACCACGUGUAACUUGGGCCAA
ACCCUAGUCUUUGUCCGUUUAGGCCCUCUGGUGCAGCUCCG

Figure 3 – Sequences for 20 individual RNA aptamer clones selected against HIV-1 RT M3 from an N100 pool after 13 rounds of selection. The constant regions are not shown, and parenthetical numbers denote repeated sequences.

repeated sequences. This follows because the length of the random region of this pool is rather long, so the possibility of a repeated sequence is not likely, especially with such a small sample size. Despite this, it was still possible for common sequence motifs to appear among the selected aptamers, so an analysis was performed using the MEME server (http://meme.sdsc.edu), which uses statistical methods in order to search for sequence families (43). Some patterns in the aptamer sequences were found, such as long strings of uridines and a lack of guanosines. However, the round 0 pool was sequenced (data not shown) in order to determine if these motifs were due to a predisposition the original pool or due to the selection process. The original pool shows the same bias against guanosine, as well as, segments of three to four uridines in a row. This fact, along with the lack of sequence homology indicates that the selection did not pick a single aptamer as an outstanding binder, but rather found a subset of aptamers that is able to bind the target well. A previous selection against mutant 3 RT was also unable to pinpoint any common motifs among the aptamers that were selected (33). Even though a common sequence motif was not found, it is not an indicator that the isolated aptamers have different structures. Rather, it is possible that the selection process evolved a structure that bound well to the target, which did not depend on a specific sequence, but rather a shape.

Characterization of Aptamer Binding and Specificity to HIV-1 RT

A cursory look at the binding of various clones to RT was assayed using a single point binding assay as was done to track the progress of the selection. The assay was performed against both wild type and mutant 3 RT, as well as, to the filter alone to check for filter binders. The data is represented in figure 4.

Although overall binding to mutant 3 was greater than wild type protein, the data cannot be taken in an absolute manner. It is possible that a portion of the wild type protein used for this assay was not viable. Even with this disparity, it is possible to take the data in a relative manner. For instance, Clone 16 bound to mutant 3 RT with a high affinity, but it did not bind to wild type RT relative to other clones. This indicates that Clone 16 is able to distinguish between the two types of RT. On the other hand, clones such as 1 and 3 bind well to both wild type and mutant 3 RT, which indicates that these aptamers do not discriminate between the two types of RT.

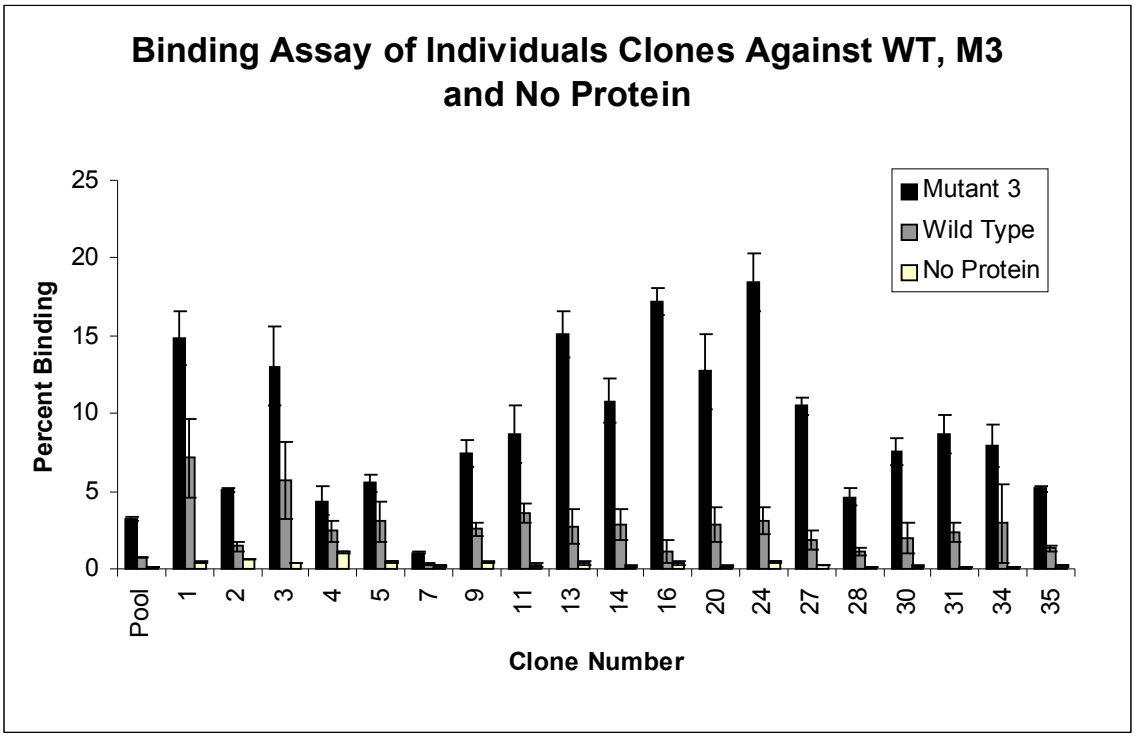

Figure 4 – Single point binding nitrocellulose binding assay for individual clones sequenced from the pool after 13 rounds of selection. Protein and RNA concentration are at 1:1 ratio of 100nM. In no protein control, the protein volume is replaced by 1X Selection Buffer

Next, the five clones with the highest percent binding against mutant 3 RT, as well as, the clone with the most repeated sequences (Clone 5) were characterized further

by obtaining their dissociation constants. The dissociation constant was determined by utilizing a binding assay where the protein concentration was varied in the presence of excess aptamer. The data is then fit into the following equation:

$$\text{RNA bound} = \frac{B_{max}\,[\text{protein}]}{K_d + [\text{protein}]}$$

From this fit, the protein concentration at which half the ligands (aptamer) bound to protein is considered the dissociation constant. The values can range from the nanomolar to millimolar scale and indicate how tightly a ligand can bind to its target, where the smaller the value, the more tightly the ligand binds. The dissociation constants for these clones are shown in figure 5. In comparsion, the canonical pseudoknot aptamers, such as TPK 1.1, bind to wild type RT with a reported K_d of approximately 5nM (26). However, TPK 1.1 was found not to bind to mutant 3 RT and had a K_d of greater than 1uM (33). Two aptamers selected specifically against mutant 3 RT, termed M302 and 12.01, were found to have K_d's of 30nM and 70nM respectively (33).

Dissociation Constants for Selected Clones Against Mutant 3 and Wild Type RT		
Clone	Mutant 3 (nM)	Wild Type (nM)
1	10 ± 2 nM	14 ± 3 nM
3	4 ± 1 nM	5 ± 1 nM
5	27 ± 1 nM	25 ± 8 nM
13	92 ± 6 nM	33 ± 2 nM
16	0.30 ± 0.04 nM	16 ± 3 nM
24	15 ± 1 nM	7 ± 1 nM

Figure 5 – Dissociation constants for 6 aptamer clones against M3 and wild type RT. Determined using nitrocellulose binding assay with a protein concentration range of 0.1nM to 1uM.

The dissociation constants for the aptamers isolated from this selection were found to bind to both wild type and mutant 3 RT with a similar affinity in most cases. The aberration was Clone 16, which bound to mutant 3 RT with a 20-fold greater affinity than

wild type protein. Clone 16 also bound to mutant 3 RT with great affinity with a dissociation constant in the mid-picomolar range (300pM). The low K_d may be attributed to a slow off rate, which has been seen in other high affinity anti-RT aptamers (44).

In order to determine which epitopes on RT these aptamers bind to, competitive binding assays were carried out (Figure 6). These assays do not determine exactly where the aptamer binds to the protein, but rather can conclude if the aptamer binds to the active site of the protein due. This is confirmed by whether or not the aptamer prevents binding of TPK 1.1 to RT, which is known to bind to the active site of the protein (24-25).

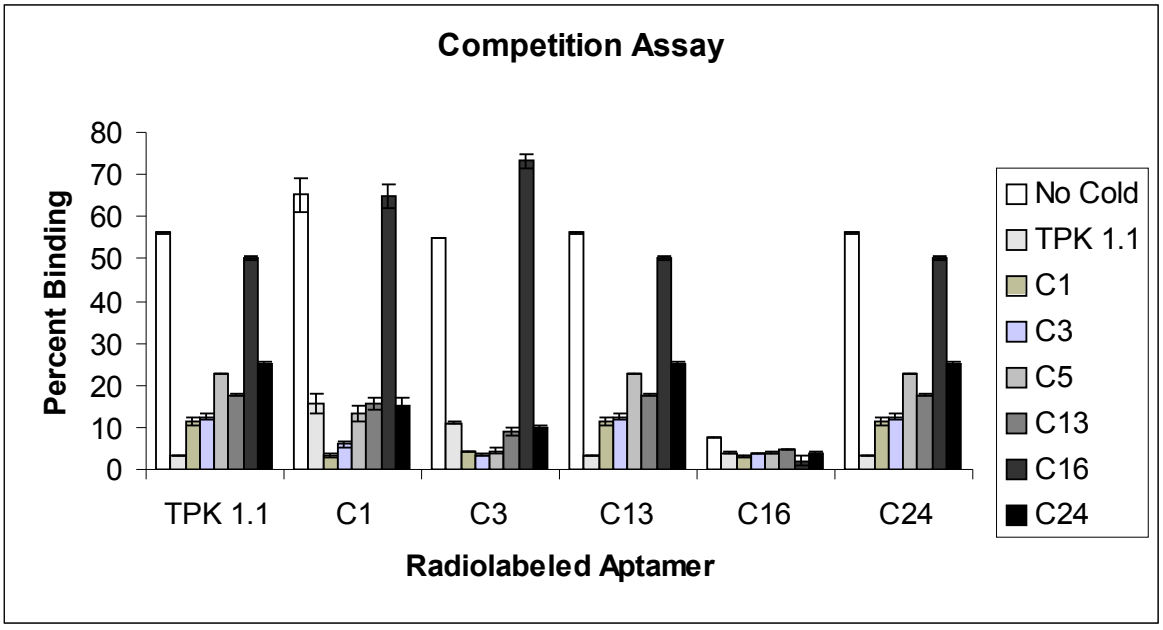

Figure 6 – Competition of selected aptamers to wild type RT using nitrocellulose binding assay. 5 clones from the selection and TPK 1.1 were body labeled at 10nM and competed against cold versions of the same RNAs at 500nM concentration. TPK 1.1 is the canonical WT RT binding pseudoknot structure used here as a positive control. HIV-1 wild type RT was at 50nM concentration.

All of the tested aptamers, except for Clone 16, competed with TPK 1.1 in binding to the active site of RT. This indicates that these selected aptamers probably bind to the primer-template cleft of the RT and should inhibit enzymatic activity. Also, these

aptamers also competed with each other, which shows that these aptamers probably bind to the same epitope on RT. The only aptamer that did not prevent the binding of TPK 1.1 was Clone 16; however, an inconsistency arose in the negative control for this aptamer. In previous K_d binding assays at 50nM wild type RT concentration, Clone 16 bound to protein at greater than 50%, but in the competition assay, Clone 16 bound to protein at less than 10%. The difference between the assays is the higher aptamer concentration used in the competition assay. The percent binding should still be similar in both assays because protein concentration is in high enough excess to ensure that the aptamer has a chance to bind its target. To look at the Clone 16 concentration dependence on binding to RT, a binding assay was conducted where protein concentration (in this case, mutant 3

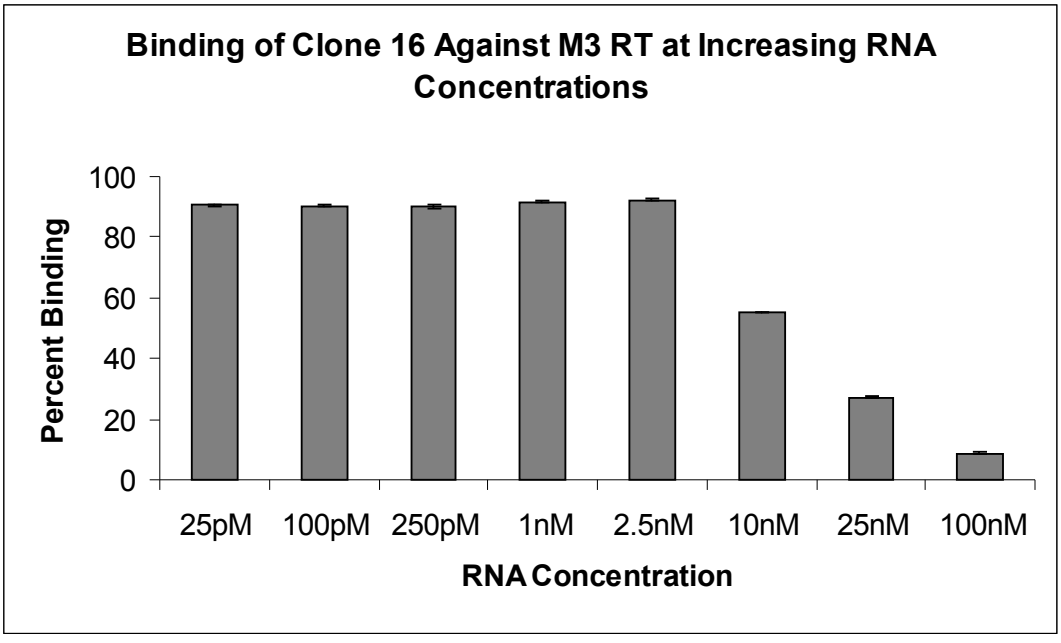

Figure 7 – Single point nitrocellulose binding assay of increasing total concentration of aptamer clone 16 (ranging from 25pM to 100nM) against mutant 3 RT at 200nM. 25pM of end labeled clone 16 RNA was used on each point and the remaining clone 16 RNA was unlabeled.

RT) was held constant at 200nM, while the Clone 16 concentration was varied from 25pM to 100nM (Figure 7). Starting from 10nM, binding of Clone 16 to mutant 3 RT decreased significantly, which indicates that aptamer may be interacting with itself to inhibit Clone 16 binding to RT.

To see if the Clone 16 aptamer interacted with itself to form a homodimer, native gel electrophoresis was utilized (Figure 8). If the aptamer did not form a homodimer, then a single band should appear representing the aptamer monomer, which is seen in the Clone 3 negative control. However, if the aptamer forms a multimer, then more than one band should appear, which is seen in the Clone 16 samples. Starting at 25nM concentration of Clone 16 aptamer, two bands start to appear and indicate that Clone 16 forms a homodimer.

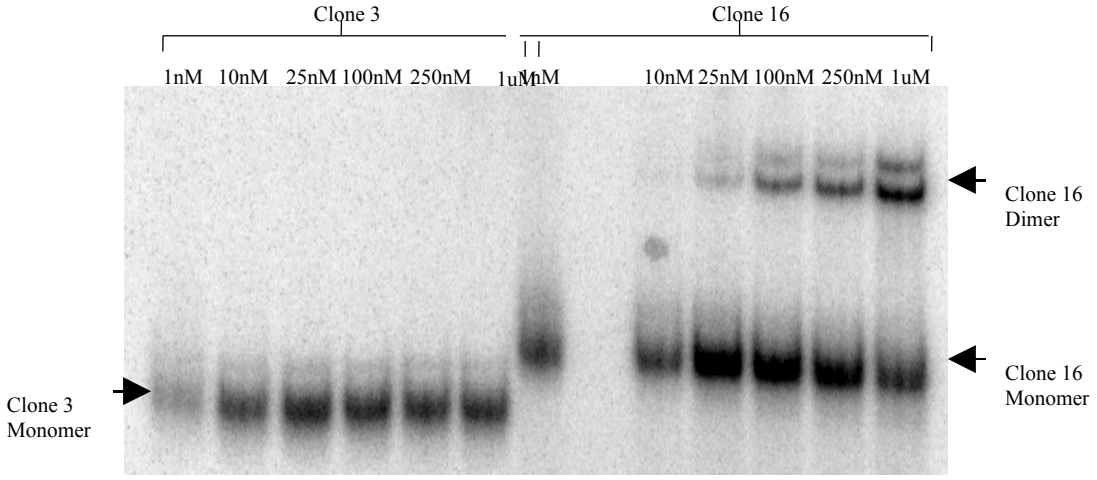

Figure 8 – Formation of homodimer by clone 16 aptamer RNA compared to non-homdimeric clone 3 RNA using native (8% non-denaturing) polyacrylamide gel electrophoresis. 1nM end-labeled aptamer and remaining cold aptamer were incubated in selection buffer at 25°C for 30 minutes.

Since the reduction in binding for Clone 16 is seen only at concentrations greater than 2.5nM, the competition assay was altered to decrease the labeled Clone 16

concentration (to 25pM) in the procedure. Additionally, the assay was performed against both wild type and mutant 3 RT to see if there is a difference in competition. Other aspects of the assay were not changed, such as protein and non-labeled aptamer concentration. The results for the assay are shown in figure 9.

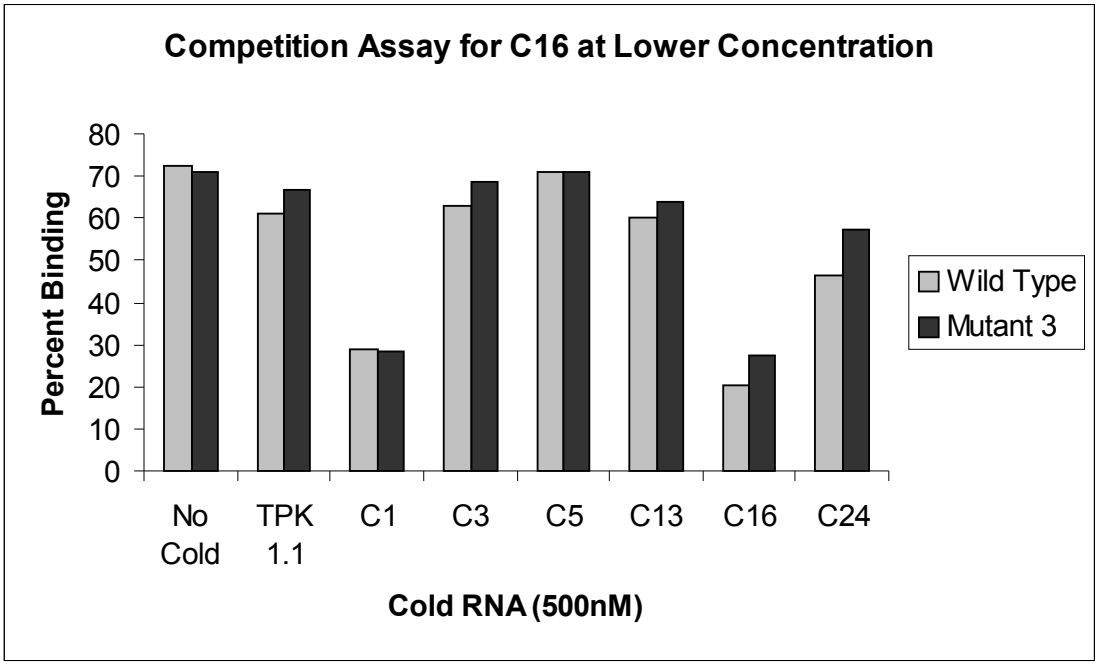

Figure 9 – Competition of aptamer clone 16 to selected aptamers on wild type RT and mutant 3 RT using nitrocellulose binding assay. Aptamer clone 16 was end-labeled at 25pM and competed against cold versions of the selected RNAs at 500nM concentration. HIV-1 wild type RT and mutant 3 RT were at 50nM concentration.

The negative control for Clone 16 (no cold aptamer) is consistent with the previous binding studies and shows the recovery of aptamer binding to RT. Clone 16 did not compete in binding with all other aptamers except for Clone 1 and Clone 16. This implies that Clone 16 probably does not bind to the same epitope on RT as TPK 1.1 and does not inhibit enzymatic activity. As expected, Clone 16 interferes with itself in binding due to competition in binding, as well as, the self-dimerization effect. The only other clone to compete with Clone 16 binding was Clone 1, which indicates that Clone 1 binds to both the active site epitope and the novel "Clone 16" epitope. With respect to the two previously selected aptamers that bound to epitopes other than the active site, neither bound to more than one of these epitopes (33). However, Clone 1 forms a structure that is able to access both of these regions on RT. Also, the assay shows that the competition effects of Clone 16 against other aptamers are the same between wild type and mutant 3 RT. This means that Clone 16 interacts with both types of RT in the same manner.

In order to see if these aptamers bound similarly to other mutant clones of RT, binding assays were carried out against mutant 5 and mutant 9 RT (8). The aptamer chosen for analysis was Clone 3 because it bound with the highest affinity to both wild type and mutant 3 RT compared to all the other characterized aptamers. The binding assay plots for Clone 3 against the wild type and various mutant RTs are shown in figure 10.

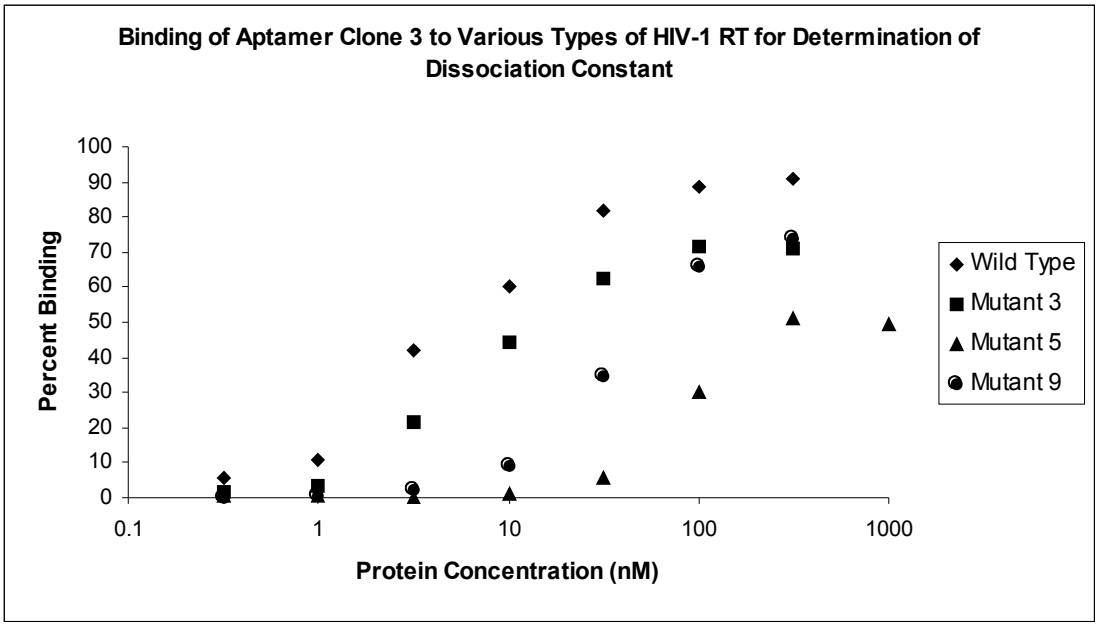

Figure 10 – Binding curve of clone 3 against Wild Type, Mutant 3, Mutant 5 and Mutant 9 RT using a nitrocellulose binding assay to determine dissociation constant. Protein concentrations ranged from 0.1nM to 1000nM and radioactively labeled aptamer was at 25pM concentration.

From the plots in figure 10, the dissociation constants for Clone 3 against mutant 5 and mutant 9 were derived (Figure 11). This aptamer binds to these two mutants with 10 to 20-fold less affinity than to wild type or mutant 3 RT. Both mutant 5 and mutant 9

Dissociation Constant for Clone 3 Against Mutant 5 and 9 RT	
Mutant 5	116 $\pm$ 40 nM
Mutant 9	50 $\pm$ 12 nM

Figure 11 – Dissociation constants for Clone 3 against mutant 5 and mutant 9 RT. Determined using nitrocellulose binding assay with a protein concentration range of 0.1nM to 1uM.

RT contain different thymidine analog mutations (TAM's) than mutant 3 RT. It is possible that one or more of these unique mutations may have interrupted key contacts between the aptamer and protein, causing a decrease in binding affinity. However, even with this decrease in binding, these aptamers still bind to these mutant RTs with a high enough affinity to have therapeutic and diagnostic utility.

Aptamers that inhibit both wild type and mutant 3 RTs

In all but one previous case, aptamers selected against HIV-1 RT seem to bind to the active site on the enzyme (24-25). Consequently, these RNA and DNA aptamers have been found to inhibit wild type RT (27). RT catalyzes many reactions and possesses such activities as DNA- and RNA-dependent DNA polymerase (DDDP and RDDP) activity and ribonuclease H (RNase H) activity (45). The majority of aptamers have inhibited the RNA-dependent DNA polymerase activity, including the canonical psuedoknot aptamer (26). This set of aptamers binds to the primer-template cleft of RT and has been called template analog RT inhibitors (TRTIs) (46). These aptamers have been found to limit polymerase activity with inhibition constants (K_i or IC_{50}) in the high picomolar to low nanomolar range (26,27,29 47). The RNase H activity has also been targeted by aptamer selections; however, these aptamers bind with a much lower affinity than their polymerase inhibiting aptamer counterparts, having inhibition constants in the low micromolar range (48-49).

Polymerase activity assays were conducted for the 6 aptamers whose dissociation constants were obtained, as well as, the initial RNA pool and TPK 1.1 (Figure 12). These assays were done against both wild type and mutant 3 RT and using three aptamer concentrations (10, 100 and 1000nM). With respect to both proteins, the initial pool and Clone 16 show no inhibition of polymerase activity. On the other hand, all selected aptamers except Clone 16 show inhibition of polymerase activity with inhibition constants that range from approximately 50 to 300nM. Also, TPK 1.1 inhibits wild type RT, but is unable to inhibit mutant 3 RT, which corroborates previous studies (33). To pinpoint a more accurate inhibition constant, more data points (aptamer concentrations) were taken for Clone 3 (Figure 13). A single clone was chosen because the five inhibiting aptamers seem to decrease polymerase activity similarly. Clone 3 was used because it is the aptamer that has been characterized to the greatest degree. The approximate inhibition constants that can be derived for Clone 3 are 70nM against mutant 3 RT and 250nM against wild type RT.

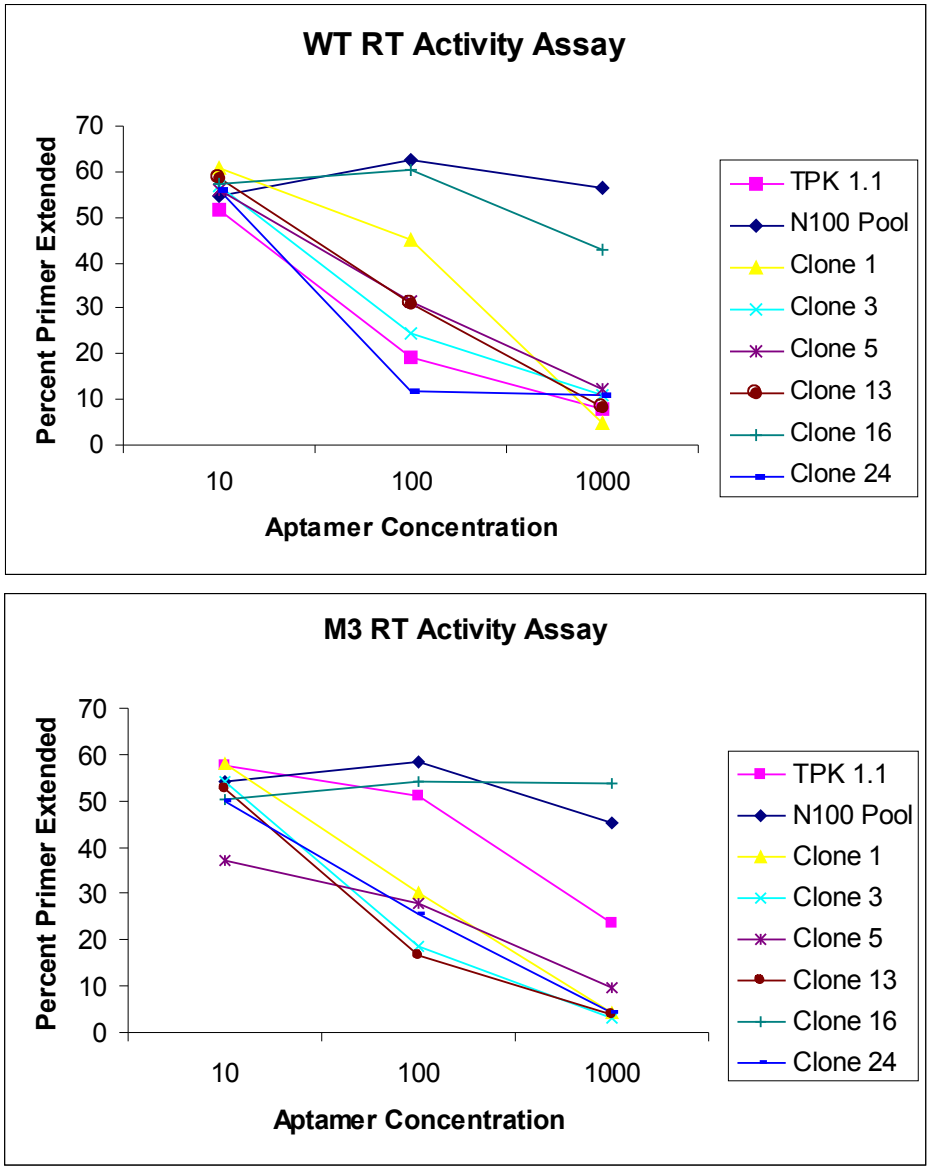

Figure 12 – Extension assays to test for polymerization activity in presence of selected aptamers at increasing concentrations. 18-nucleotide length primer was end-labeled and incubated at 37°C for 10 minutes with 10nM 100-nucleotide RNA template, 50nM wild type (top) and mutant 3 (bottom) RT and 50uM dNTP's. Percent of primer that has been extended to 100 nucleotides in presence of various aptamers is shown in graph.

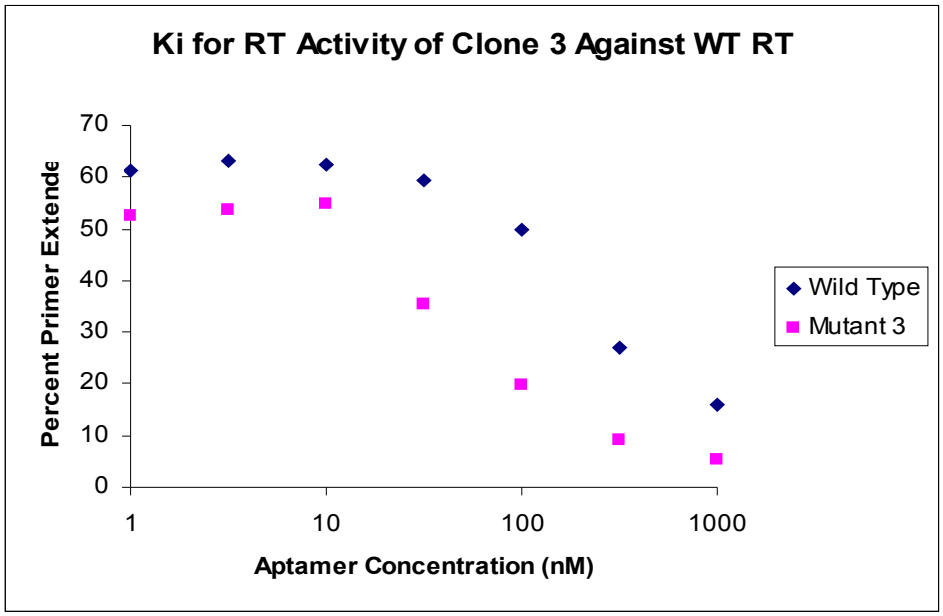

Figure 13 – Polymerization activity assay of wild type and mutant 3 RT in presence of increasing aptamer clone 3 concentration (ranging from 1 to 1000nM) for determination of inhibition constant.

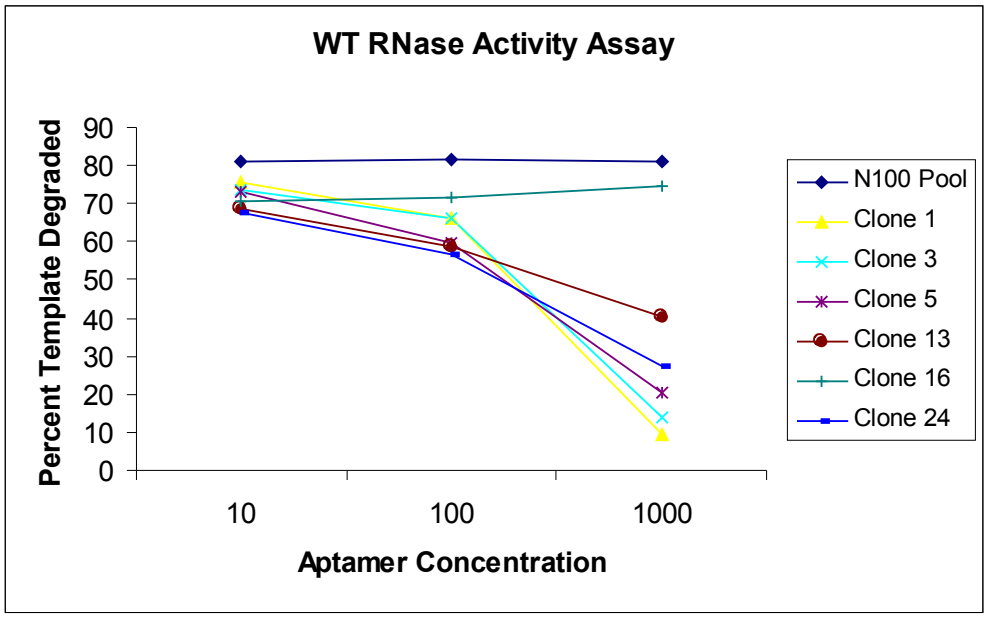

Figure 14 – RNase H activity assays to test for RNA digestion activity in presence of selected aptamers at increasing concentrations. 100-nucleotide length RNA template was end-labeled and incubated at 37°C for 10 minutes with 10nM 18-nucleotide DNA primer and 50nM wild type RT. Cleaved product is 82-nucleotides long as 18-nucleotide region that base pairs with primer is degraded. Percent of template that has been cleaved to 82 nucleotides in presence of various aptamers is shown in graph.

Ribonuclease H (RNase H) activity assays were also conducted against the same set of aptamers used for the polymerase activity assays. Again, three concentration points were taken for all aptamers (Figure 14), and then a full set of points was taken for Clone 3 (Figure 15). However, only wild type RT was tested for activity because mutant 3 RT lacks RNase H activity, as shown in previous studies (33). The assay shows that RNase H activity is being inhibited by the same aptamers that inhibited polymerase activity. However, the concentration at which half of RNase H activity is inhibited is much higher than the concentration for polymerase activity inhibition. For instance, the inhibition constant for polymerase activity of Clone 3 against wild type RT is 70nM, while the inhibition constant for RNase H activity is approximately 250nM. This may

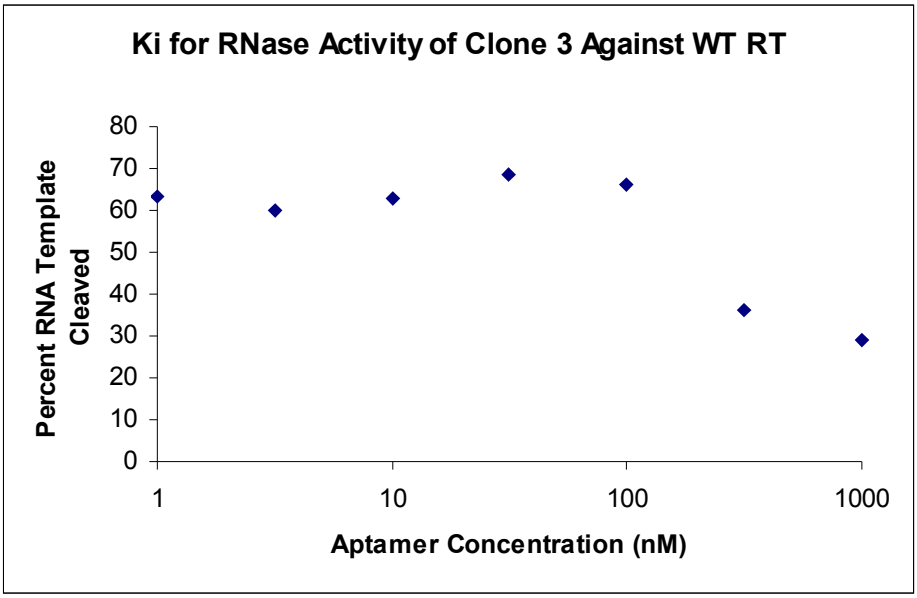

Figure 15 – RNase H activity assay of wild type in presence of increasing aptamer clone 3 concentration (ranging from 1 to 1000nM) for determination of inhibition constant.

imply that the mechanism for inhibition links the two activities together. Hypothetically, since the primer-template duplex cannot bind to RT due to the presence of aptamer binding to the active site, then as a consequence, the RNase H domain cannot access the

template in order to cleave it. This type of inhibitory effect has been seen in previous studies as well (33).

From these assays, Clone 16 was found to inhibit neither activity of RT and did not compete with the other aptamers in binding to RT. As a result, it can be concluded that Clone 16 binds to a unique epitope on RT. Also, the other inhibitory aptamers, such as Clone 3, can inhibit the polymerase activity, as well as, the RNase H activity to a lesser degree, for both wild type and mutant 3 RT. These aptamers were able to overcome the mutations that prevent TPK 1.1 from binding to mutant 3 RT, but still bound to and inhibited wild type RT.

Building Aptamer Complexes Bind More Tightly to Target Protein

Clone 16 was found to bind to an epitope separate from the active site of RT. Since Clone 16 also bound to RT with a very high affinity, it is the perfect candidate to link to another aptamer, which binds to the active site of the enzyme. Hypothetically, Clone 16 could function as a tether to assist TPK 1.1 in binding to 3 RT, and consequently, Clone 16 can assist in inhibiting polymerase activity. There are many methods to link two aptamers together. The method chosen was to add extensions to each aptamer and have a DNA linker hybrize to those extensions (Figure 16)

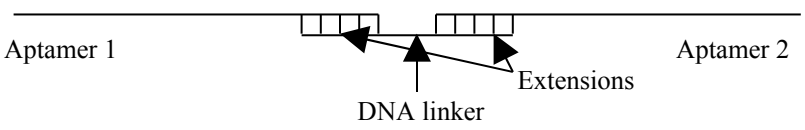

Figure 16 – Scheme for the linking of two aptamers together. A DNA linker is used to bind to extensions that have been added to the 3' and 5' end of aptamer 1 (Clone 16) and aptamer 2 (TPK 1.1) respectively.

To see if the method created proper complexes, native gel electrophoresis was utilized (Figure 17). Clearly from the assay, there is a discrete shift after each "part" of the complex is added into the sample. The top band in lane 4 and lane 7 represent the

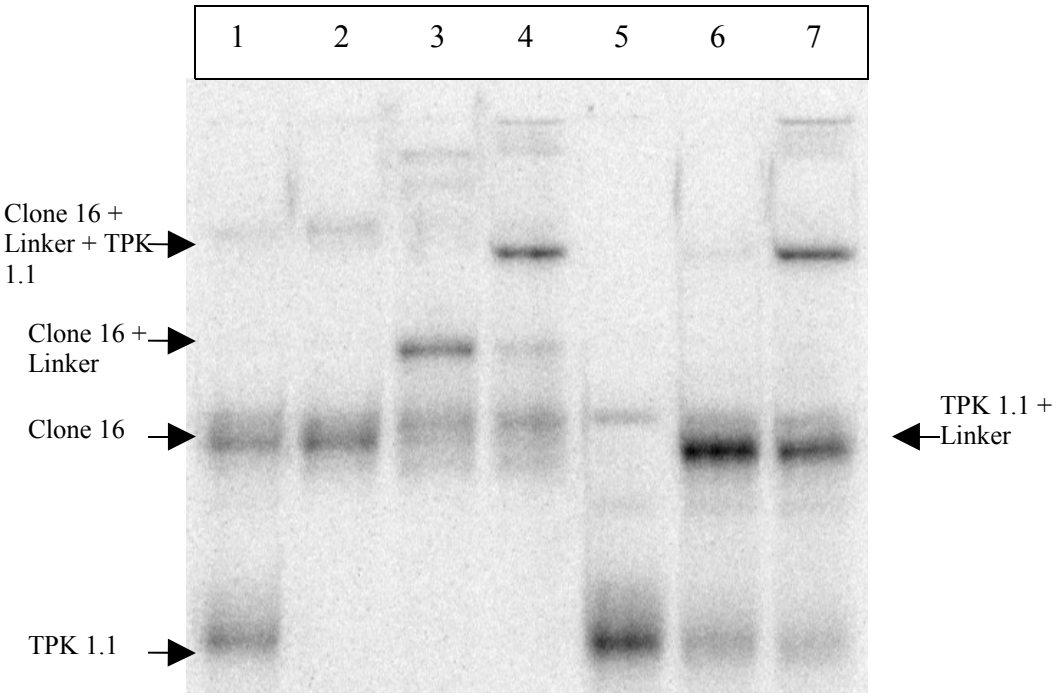

Figure 17 – Native gel electrophoresis to look for complex formation by detecting shifts in bands. The concentration of each "part" is 100nM. The lanes are as follows: (1) Clone 16 + TPK 1.1 (2) Clone 16 (3) Clone 16 + Linker (4) Clone 16 + Linker + TPK 1.1 (5) TPK 1.1 (6) TPK 1.1 + Linker (7) TPK 1.1 + Linker + Clone 16.

two aptamer complex. However, there is not a complete shift due to inefficiency in hybridization. Additionally, after preliminary binding studies, it was determined that the linker was a filter binder. Using end-labeled primer at 25pM and no protein, it was found that greater than 90% of the linker bound to the filter (data not shown).

To see if this filter binding could be reduced by ensuring that all linker bound to the labeled aptamer is also bound to the unlabeled aptamer, a binding assay was done where the concentration of the labeled aptamer and linker were kept constant, while the concentration of the unlabeled aptamer was varied (Figure 18). From the data, filter

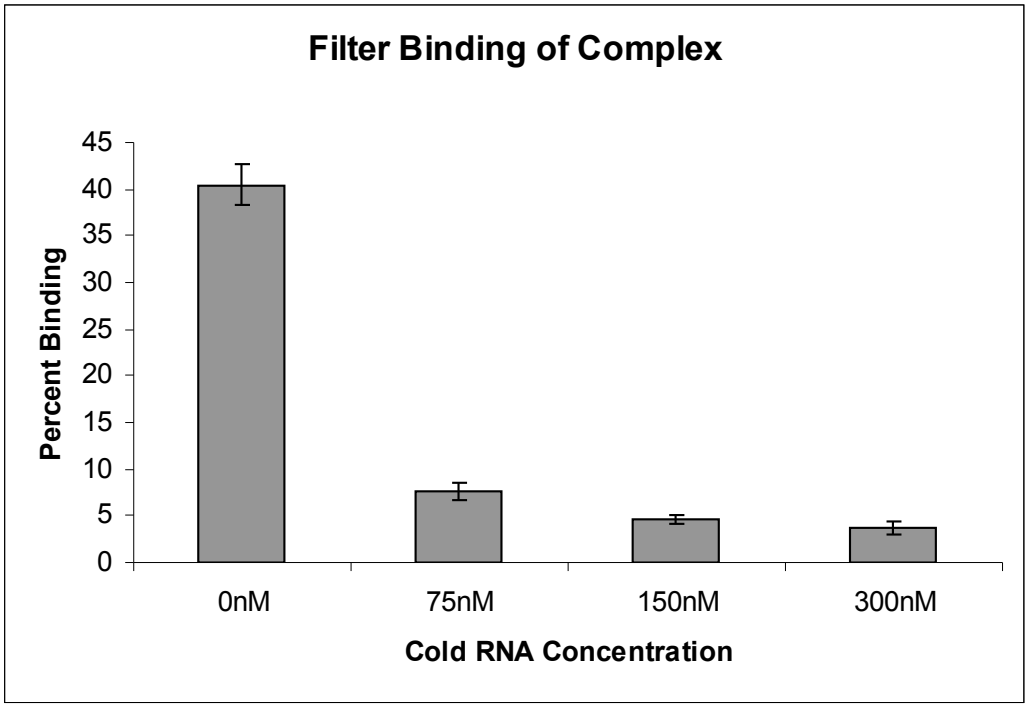

Figure 18 – Binding assay to determine amount of complex that binds to filter, so protein was replaced by 1X selection buffer. Concentration of labeled TPK 1.1 was 25nM and concentration of linker was 50nM. The concentration of unlabeled Clone 16 was varied between 0 and 300nM.

binding was reduced from 40% to less than 5% at 300nM Clone 16 concentration.

However, utilizing such a high Clone 16 concentration may cause homodimer formation, even when it is in complex with the linker oligonucleotide. Using native gel electrophoresis, it was seen that this was indeed the case (Figure 19). Lane 3 shows the formation of various bands due to Clone 16 interacting with itself, even in the presence of the linker. So even though the filter binding problem was solved by increasing Clone 16 concentration, another difficulty arose with Clone 16 interacting with other Clone 16 aptamers in solution. Consequently, it seems that this scheme for linking aptamers together is not viable. A better option may be to directly link the two aptamers together as a single construct. Even though this method did not work for this set of aptamers, it is

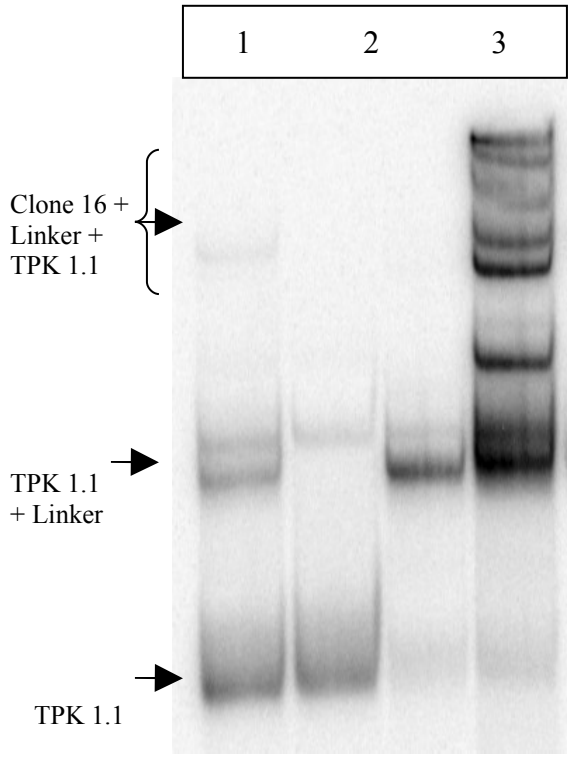

Figure 17 – Native gel electrophoresis to look for complex formation by detecting shifts in bands. The concentration are as follows: TPK 1.1 is 25nM, Linker is 50nM and Clone 16 is 300nM. The lanes are as follows: (1) TPK 1.1 (2) TPK 1.1 + Linker (3) TPK 1.1 + Linker + Clone 16.

still a viable option for other sets of aptamers due to its robust nature because it able to use the same linker for different aptamer pairs or the linker length can be altered without requiring new extensions on the aptamers. But this set of experiments show that the aptamer pairs need to be chosen carefully and not just based on the fact that they bind to separate epitopes on the same protein.

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