Switchable Aptamers (SwAps) for the Purification of Vesicular Stomatitis Virus (VSV)

Mohamed Wehbe

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Abstract

Oncolytic virus therapy for the treatment of cancer has received a lot of positive attention as a promising cancer remedy. Vesicular Stomatitis Virus (VSV) is one oncolytic virus which has been considered twice for clinical trial for its selective destruction of cancer cells. (U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2008)(U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2008) For oncolytic virus therapy to be feasible, obtaining pure virus samples is necessary as it can only be administered as a sterile injection. Here we offer a novel method of affinity purification using switchable aptamers (SwAps) as a means to meet the requirements necessary for the use of oncolytic viruses. Aptamers are single stranded (ss) DNA/RNA that form tertiary structures which allow them to bind to their targets with high affinity and specificity.(Jayasena, 1999) They were previously selected for VSV in Dulbecco's phosphate buffered saline (DPBS) and showed strong binding affinity. It is believed that high concentrations of Mg^{2+} and Ca^{2+} ions that are present in this solution chelate ssDNA to form more rigid tertiary structures. (Nomura, et al., 2010) Hence, DPBS allows for better aptamer-virus binding because it contains $MgCl_2$ and $CaCl_2$. Through a modified cell-SELEX procedure, we have exploited the presence of these divalent cations and developed SwAps that allow for control over aptamer-VSV association and dissociation. This is done by including an additional step in the SELEX process which involves EDTA and EGTA to sequester Mg^{2+} and Ca^{2+} ions. This results in a conformational change in the aptamer causing the release of the virus which can then be collected. Upon 13 rounds of selection we have found two pools that show high VSV affinity and switchable functionality. Rounds 3 and 10 both proved to be strong switchable aptamer pool candidates and round 10 was selected for cloning and sequencing.

Acknowledgement

I'd like to thank Dr. Maxim Berezovski for his guidance over the past two years. I cannot express how much I appreciate the time he has spent with me and the effects he has had on me as a scientist and researcher.

I would like to express my gratitude to Dr. Anya Zamay who has truly been a mentor to me over this year. I'm thankful for your constant support and for answering my constant barrage of emails even after you returned to Russia. I feel honoured to have met you and feel that I've become a better scientist by working with you.

I would like to show my appreciation to Darija Muharemagic for the amount of time she has spent with me in the flow cytometer room and for providing your insight during our lengthy discussions.

I'd like to thank Salma Iqbal for her help with cell culture and Afnan Azizi for his work involving VSV contamination. Also, I would like to thank Ana Gargaun and Nasrin Khan for their support in the lab.

Statement of Contribution

Conception:

The idea of switchable aptamers was conceptualized by Dr. Maxim Berezovski, Darija Muharemagic and Mohamed Wehbe. The SELEX protocol was adapted from Dr.Anya Zamay's selections and altered by Maxim Berezovski.

Experimental:

The initial aptamer pool was donated by Dr.Anya Zamay. All subsequent rounds of selection were performed by Mohamed Wehbe. Training for Beckman FC500 flow cytometer was given by Dr.Anya Zamay and Darija Muharemagic. All affinity analysis testing was done by Mohamed Wehbe. Vesicular Stomatitis Virus was obtained from Dr. John Bell lab for round selection and then was harvested by Mohamed Wehbe.

Writing

This thesis was written by Mohamed Wehbe and edited by Salma Iqbal and Darija Muharemagic

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Introduction:

Vesicular Stomatitis Virus (VSV) has shown potent oncolytic virus (OV) activity against many tumour types. (Siddharth, Porosnicu, & Barber, 2001). One of the challenges is to develop a method of purification that is quick and simple, as it can only be used for clinical applications if it is free from contaminants. We offer a novel method for affinity purification using switchable aptamers (SwAps) as a mean to solve this problem and allow for the continued study into this OV.

Cancer

Despite advances in diagnostic techniques and treatment methods cancer is still one of the leading causes of death around the world.(GLOBOCAN, 2008) Cancer is a broad term used to encompass many diseases. In general, it is a compilation of mutations inside a cell resulting in uncontrollable growth. (Hartwell & Kastan, 1994)The mutations can be spontaneous (error in DNA replication) or induced (radiation, chemicals, etc.) and it is impossible to associate a cancerous mutation in the body with any one mechanism. (Brenner, Rothenbacher, & Arndt, 2009)(Pagano, et al., 453-471)(Samaras, Rafailidis, Mourtzoukou, Peppas, & Falagas, 2010) There have been many treatment options approved for cancer; these range from physical removal of tumour to the destruction of cancerous cells using small molecule drugs or radiation therapy.(Kufe, et al., 2003)Each of these therapies has their own respective risks and drawbacks. Surgical removal of tumours is limited largely by the location of the tumour. During surgical removal, an excess amount of normal tissue must be removed to ensure the complete elimination of the tumour tissue. Chemotherapy is also common for cancer treatment, especially when the cancer has metastasized and spread around the body.(Kufe, et al., 2003) However,

chemotherapeutics have very low selectivity and destroy all fast growing cells in the body. Furthermore, it is understood that upon rounds of chemotherapy, cancer cells mutate to avoid destruction by the drug but the body's natural defenses are weakened (lowered immunity).(Gottesman, 2002) Unfortunately, this results in the inability to continue chemotherapy and the patient runs out of options unless the immune system can recover. Thus, cancer therapy begs for new methods of treatment that have cancer cell specificity as well as low adverse effects to the patient.

Oncolytic virus (OV) therapy is an emerging treatment for cancer discovered in 1912. It was discovered that some patients with viral syndromes showed rare and dramatic reduction in cancer. (Hansen & Libnoch, 1978)(Bousser & Zittoun, 1965) OVs are competent DNA or RNA viruses that selectively replicate in cancer cells. This new method of treatment has been shown to be relatively safe and generate clinical response in tumours that are otherwise unresponsive to chemotherapy and radiotherapy. (Pecora, et al., 2002) These benefits have further driven OV research as it is an innovative alternative for cancer treatment. OV specificity arises from modifications of known viruses. (Frentzen, et al., 2009) There are many OVs which have been modified for oncolytic activity including adenovirus (Kuruppu & Tanabe, 2005), paramyxovirus (Voroshilova, 1989), herpes simplex virus (Pond & Manuelidis, 1964), reovirus (Kunin, 1964), poxvirus and rabies virus (Eager & Nemunaitis, 2011), many of which are currently in clinical trials. This thesis involves the purification of an OV from the family *Rhabdoviridae;* Vesicular Stomatitis Virus (VSV) Δ51 has shown strong oncolytic activity as it was found to be efficient at reducing tumour size when used in either intratumoural or intravenous injections by showing the ability of proliferating selectively in tumour cells.(Lun, et al., 2006) Thus, showing great promise as a therapy to treat many different types of cancer.

Vesicular Stomatitis Virus

Dr. John Bell and Peter Forsyth discovered a variant of the vesicular stomatitis virus (VSV) which has shown oncolytic activity. (Lun, et al., 2006)VSV is a negative single strand RNA virus and is arthropod-borne from the *Rhabdoviridae family* with a viral genome of 11kbp. (Lichty, Power, Stojdl, & Bell, 2004)One of the benefits of using a RNA virus that replicates in the cytoplasm is that it does not risk causing mutations in the host cell DNA [which could cause cancer]. During infection, VSV synthesizes five distinct proteins required for viral replication and escapes from cell defences; these proteins are known as nucleoprotein, phosphoprotein, glycoprotein, matrix protein and large polymerase protein.(Wagner & Rose, 1996)The nucleoprotein, phosphoprotein and large polymerase protein, along with host proteins, are responsible for virus transcription and replication in the cytoplasm.(Lichty, Power, Stojdl, & Bell, 2004) To replicate, the virus must first synthesize a positive strand RNA complementary to the negative as this strand will be replicated to create more negative strands required for VSV growth and proliferation. The glycoprotein is crucial for viral binding to target cells and is necessary for VSV fusion to cell membranes and release of virus into the cytoplasm.(Whelan, Barr, & Wertz, 2004) The matrix protein consists of 229 amino acids and is used in virus assembly, budding, cellular apoptosis and disruption of host-cell innate immunity programs.(Lichty, Power, Stojdl, & Bell, 2004) Furthermore, cellular apoptosis is an important step in the virus replication cycle as this is when the virus gains its envelope which is taken from the host cell membrane. The matrix protein is imperative to the immunity of the virus and is thus the key to selective oncolytic virus activity.

Dr. John Bell developed two variants of VSV that showed sensitivity to the interferon pathway and could potentially be used for oncolytic virus therapy. These mutants, named AV1 and AV2 were sequenced to see the variation between them and the wild type VSV; it was found that they were both a result of amino acid substitutions in their M protein.(Stojdl, et al., 2000) AV1 is substituted at M51R and AV2 at V221F and S226R, both of these mutations inhibited the antiviral ability of VSV; thus, a third variant (AV3) was created to mimic AV1 by deleting M51R (known as VSVΔ51). The biological and antiviral properties of VSVΔ51 are identical to those of AV1 and AV2. The mutants were studied further to elucidate the mechanism of action and it was found that upon infection both mutant and wild type trigger the primary response genes in the cell. Upon performing Western blot analysis it was seen that IFN-beta from the secondary and IFN-alpha from the tertiary steps were seen in the mutants but not in the wild type. Thus, wild type VSV disables host cell antiviral response by disrupting activation of secondary and tertiary response steps. It is hypothesized that in wild type VSV the M protein is able to inhibit nuclear export of antiviral mRNA from cell nucleus.(Stojdl, et al., 2000)

One benefit of using VSV as an OV is that only a small population in the entire world has immunity against the virus.(Lichty, Power, Stojdl, & Bell, 2004) With long term oncolytic virus therapy, the host system develops immunity against the virus which results in the removal of the virus before oncolytic activity. To prevent destruction and removal by host immune system many methods have been employed; this includes polymer-coating technologies with poly-[N-(2-hydroxypropyl) methacrylamide] (HPMA) and polyethylene glycol (PEG), pre-infected T cells as carriers for delivery of oncolytic viruses to tumor sites, or aptamer based protection.(Labib, et al., 2011) Another large problem with OVs is obtaining pure virus samples for injection. As VSVΔ51 becomes increasingly in demand as an OV, the need for a method to obtain large

quantities of pure virus also increases. Upon infecting cells with VSVΔ51, purification involves many centrifugation and washing steps to remove cell debris.(Diallo, Vähä-Koskela, Le Boeuf, & Bell, 2012) Unfortunately, increasing washing steps leads to low yields of pure intact virus, whereas decreasing washing steps leads to large quantities of cell debris and other contaminants.

Purification Methods:

There are two main groups of purification methods used for OVs: density gradient centrifugation and heparin column purification. The density gradient methods include iodixanol (OptiPrep^{TM}) and sucrose gradients. In both methods, purification is performed by forming a gradient of differing densities; upon centrifugation of the virus and contaminating cell debris the virus remains at a point where its density matches that of the gradient and then can be collected. (Diallo, et al. 2012)This method is not very expensive but does leave some cellular residue in the solution. Furthermore, it should be noted that the sucrose gradient technique has been proved to decrease the infectivity of viruses.(Møller-Larsen & Christensen, 1998) On the other hand, the heparin column purification employs the use of sepharose beads conjugated to heparin molecules. This is typically used to purify proteins but can also be employed for viruses; this method yields a purer product when compared to the density gradient method. (Segura, Kamen, Trudel, & Garnie, 2005) Unfortunately, heparin column purification is very expensive. Thus, what is required is a method which is less expensive but still offers the high level of purity which comes from affinity purification. Here we offer a novel approach which incorporates affinity purification through reusable aptamers thus resulting in a low-cost method.

Aptamers

Aptamers are short single stranded DNA/RNA oligonucleotides that are capable of specific and strong binding to their target molecule. This occurs due to the presence of stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplexes in the tertiary structure. (Strehlitz, et al.2012) Typically, they are 40-100 nucleotides longs and contain three distinct portions: a random region of nucleic acids, flanked by forward and reverse primer regions. A diverse range of target molecules have been used in aptamer selection including organic dyes (Ellington & Szostak, 1990), amino acids (Tuerk & Gold, 1990), antibiotics (Tuerk and Gold, 1990), proteins (Daniels, et al. 2003), whole cells (Phillips, et al. 2008) and viruses(Labib, et al., 2011). Selection of aptamers is most often done through a process called SELEX (systemic evolution of ligands by exponential enrichment), commencing with a library of synthetic DNA which can contain up to 10^{15} oligonucleotide sequences. (Cho, Lee, & Ellington, 2009) It is expected that some members of the library will bind to the target and thus, in each successive round of selection, one must efficiently separate those few binders and amplify them with PCR. The selection is performed entirely *in vitro* and has the potential of being completely automated. (Cox, Hayhurst, Hesselberth, Bayer, & Georgiou, 2002)

Switchable Aptamers

Aptamers were previously selected for the purpose of protecting the virus; this is known as Aptamer Virus Immuno-Shielding (AptaVISH). (Labib, et al., 2011) Although, these aptamers have the potential to solve the problem of viral destruction by host immune system, the problem of purification is still present. Aptamer selection is typically performed in Dulbecco's phosphate buffered saline (DPBS) which contains $MgCl_2$ and $CaCl_2$ respectively. The Ca^{2+} and Mg^{2+} ions help stabilize the phosphate backbone through chelation and thereby increase the tertiary

structure stability. (Hart, 2000) Thus, it stands to reason that by disrupting chelation of Ca^{2+} and Mg^{2+} to the backbone, one can also disrupt binding of aptamer to the virus. This is the idea behind switchable aptamers (SwAps). This involved the use of EDTA and EGTA to leach $Mg^{2+}$and Ca^{2+}, which was expected to cause a conformational change in the aptamer's tertiary structure which would release the virus. (Figure 1) The resulting aptamers have a new functionality where one could alter their binding capabilities by removing the chelating ions. This can be incorporated to aide in the problem of viral purification. Thus, to purify VSVΔ51, one could hypothetically modify a method normally used to select aptamers which involves the use of streptavidin coated magnetic beads and biotinylated aptamers. (Berezovski, et al. 2008) (Figure 2) A mixture of unpurified VSVΔ51could then be added; upon addition, aptamers would bind selectively to the virus. One could wash the beads to remove contaminating particles and then add EDTA/EGTA to release the virus which could be collected and used for OV treatment. This offers a cheap method of affinity purification, which in turn offers VSVΔ51 that can be used for OV therapy.

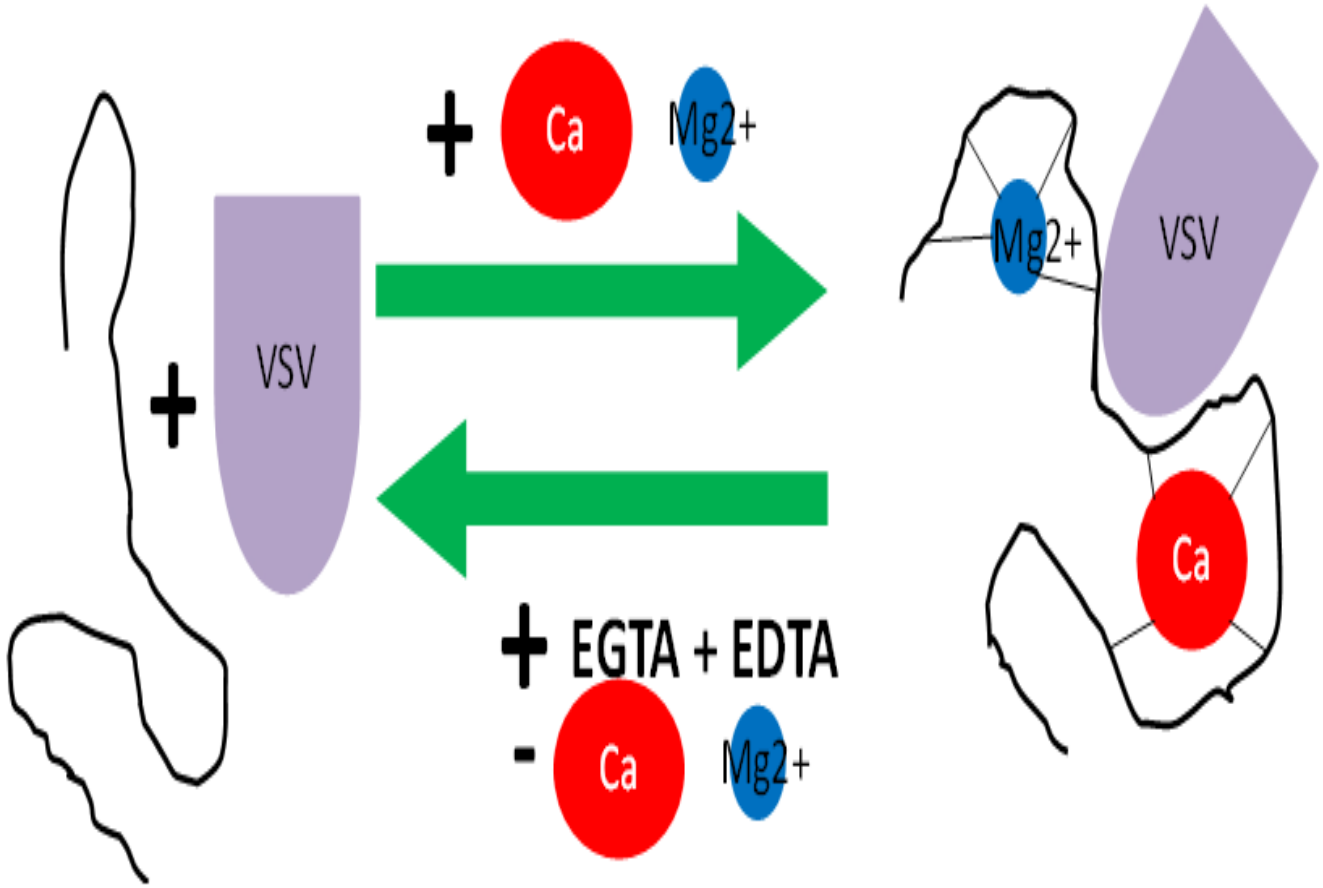

Figure 1: The binding of Switchable aptamers to VSV. In the presence of Mg^{2+} and Ca^{2+}, aptamers bind to VSV. Upon removal of the metals through the addition of 2.5 mM of EGTA and EDTA, a conformational change occurs which releases the virus.

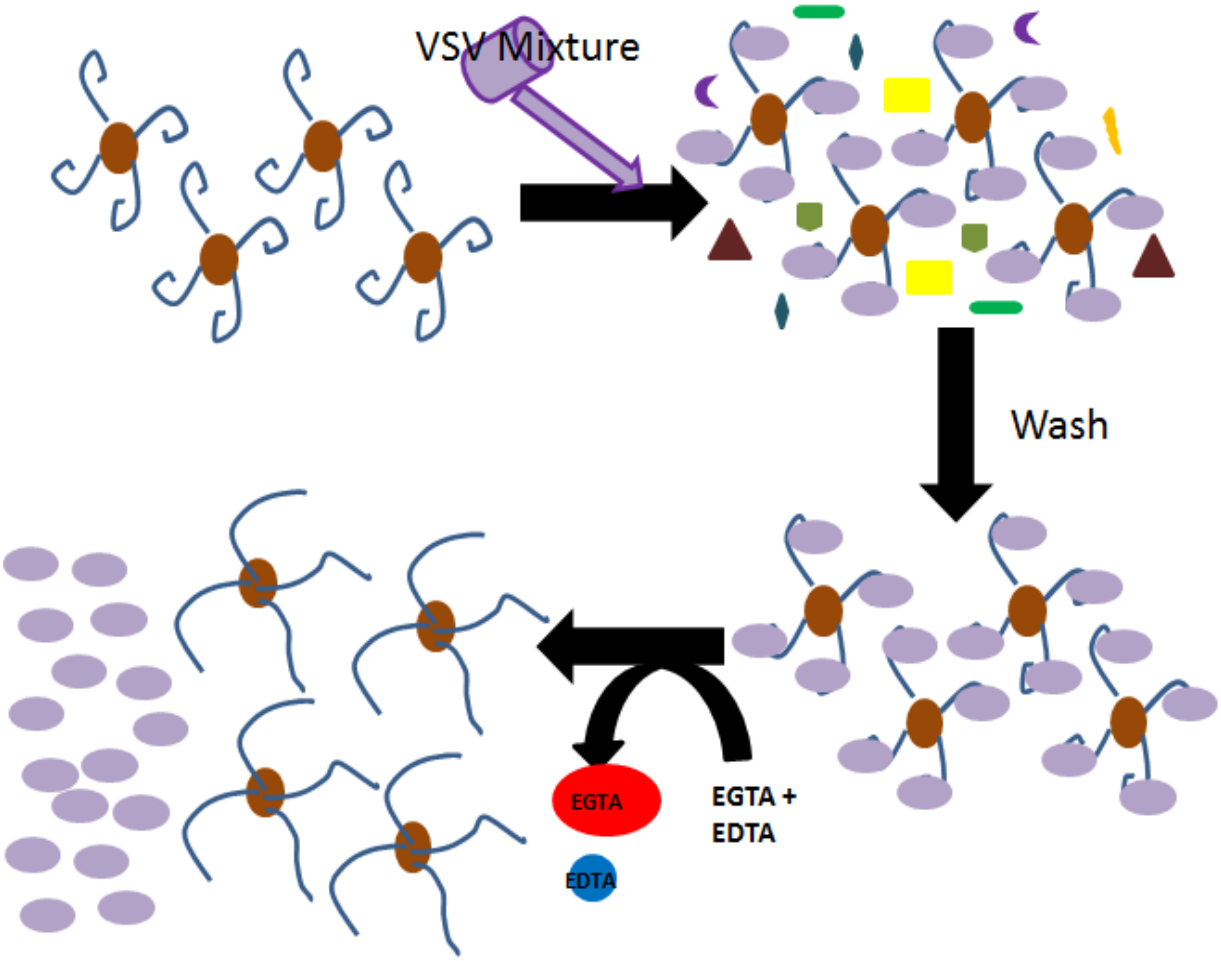

Figure 2: Virus purification method using switchable aptamers. (a)Biotinylated aptamers are attached to streptavidin coated magnetic beads. (b)Incubated with virus mixture containing cell debris; aptamer-virus complex is formed. Beads are washed to move contaminants. 2.5mM EDTA/EGTA is added causing the conformational change which releases the virus. I would put little letter next to each step and then you could refer to them before each sentence.

Materials and Methods

DNA Library:

N40 DNA library (Integrated DNA Technologies Inc.) was used for all experiments. Library members, single stranded DNA are 80 oligonucleotides in length. Consist of two flanking primer regions of 20 nucleotides each. The center portion is the random nucleotide region which is 40 nucleotides in length. .

Selection of Switchable Aptamers to VSVΔ51 (rounds 1-10):

Aptamer round 8 preselected for VSVΔ51 was obtained from Dr. Anya Zamay. (Labib, et al., 2011) Aptamer pool was denatured by heating at 95 °C for 5 mins in DPBS (D8662, Sigma-Aldrich) and allowed to re-fold on ice for 10min. $2.5x10^9$pfu/mL ofVSVΔ51 (Donated by Bell Lab) was incubated with 100nM of FAM-labeled aptamer pool 8 for a total volume of 50 µL in DPBS for 30 minutes on shaking incubator 400 rpm at 25^oC. Mixture was then centrifuged at 17200rcf for 15minutes. Supernatant was discarded and 50uL DPBS was added and mixture was centrifuged again. This washing step was repeated 3 times for rounds 1-5 and increased to 5 times for rounds 6-10. Upon completion of last washing step, pellet was re-suspended in 50 µL of 2.5 mM EDTA (EMD Chemicals) / EGTA (Bio Basic Inc.) in PBS and allowed to incubate for 30 minutes. The mixture was centrifuged for 15minutes at 17200rcf and the supernatant was transferred into a separate tube for storage at -20^oC. Aptamers were amplified by PCR and the cycle was repeated.

Selection of Switchable Aptamers to VSVΔ51 (rounds 11-13):

100 nM of FAM-labelled aptamer round 10 was used to continue selection with $2.5x10^9$ pfu/mL of VSVΔ51.The solution was incubated for 30 minutes on shaking incubator at 25^oC. Mixture was centrifuged at 17200 rcf for 15minutes. Supernatant was discarded, 50uL DPBS

was added and mixture was centrifuged again. This washing step was repeated 5 times. Upon completion of last washing step, pellet was re-suspended in first 50 µL of 500 µM EDTA/EGTA in PBS and allowed to incubate for 30 minutes. The mixture was centrifuged for 15 minutes at 17200 rcf and the supernatant was transferred into a separate tube for storage as the low EDTA/EGTA concentration fraction (l). The remaining pellet was then incubated with 2.5 mM for 30 minutes to make the medium concentration fraction (m) upon centrifugation and collection of supernatant. Finally, 10mM EDTA/EGTA was added to the pellet and upon incubation for 30 mins and centrifugation the high concentration fractions was collected. Aptamers were amplified by PCR and the cycle was repeated for each low, medium and high fraction in parallel.

PCR:

Aptamer pools were amplified using symmetric and asymmetric PCR cycles after each subsequent round of selection. Symmetric PCR synthesizes dsDNA; 5µL of the supernatant collected during selection containing the bound aptamers was mixed with 45 µL of symmetric PCR master mix. The master mix contained the following reagents in final concentrations of:: 1× PCR buffer (Promega Corporation), 2.5mM MgCl2, 0.028 U µL−1 GoTaq Hot Start Polymerase (Promega Corporation), 220 µM dNTPs, 500 nM forward primer (5′-CTC CTC TGA CTG TAA CCA CG-3′) (Integrated DNA Technology), and 500 nM reverse primer (5′-GGC TTC TGG ACT ACC TAT GC-3′) (Integrated DNA Technology). Upon completion, 5 µL of symmetric master mix was added to the asymmetric PCR master mix containing the same reagents as the symmetric master mix but with 1µM forward FAM-primer (5′-FAM-CTC CTC TGA CTG TAA CCA CG-3′) and 50 nM reverse primer. Amplification was performed for both symmetric and asymmetric PCR using the following program: preheating for 2 min at 95 °C, 15cycles for

symmetric PCR or 10−15 cycles for asymmetric PCR of 30 sec at 95 °C, 15 s at 56.3 °C, 15 s at 72 °C, and hold at 4 °C.

Aptamer Purification:

For affinity testing pools were purified by loading the mixture on 30 kDa cut-off filter (Nanosep, PALL). This was then centrifuged at 3800 rcf for 13 minutes at 16^{o}C, this was followed by the addition of equal volume DPBS for two additional washing steps for 10 minutes. The purity was tested by running raw and purified samples on 3% agar gel (Sigma Aldridge) at 150V. Finally, concentration of sample was measured using NanoDrop-2000 UV−Vis spectrophotometer.

Flow cytometry:

Aptamer pool affinity to VSVΔ51 and switchability was measured using a FC-500 Flow Cytometer (Beckman Coulter Inc.). All samples contained 100 nM of purified FAM labeled aptamer pool and were incubated with 2.5x10^{7} PFU/mL at room temperature for 30 minutes in DPBS. The samples were then divided into two portions; the first portion had DPBS added to it the second had 2.5 mM EDTA/EGTA. The second portion was allowed to incubate with the EDTA/EGTA for 30 minutes at room temperature. All samples were made to 250 µL prior to flow analysis. Control experiments were performed using the initial aptamer pool 8 and a sample of VSVΔ51 was stained using TOTO-3 dye (Invitrogen) to allow for identification on flow cytometry.

Harvesting and Purification of VSV:

The protocol for harvesting VSV as was previously described.(Diallo, Vähä-Koskela, Le Boeuf, & Bell, 2012) Briefly, 6 plates of Vero cells were grown until confluent and then were infected with VSV-Δ51 (approx. 10^{6}pfu/ plate). After 24hr supernatant was collected into 50mL

tubes and were pre-cleared of cell debris through centrifugation. The supernatant was passed through 0.2 µm filters (Pall). The pellet containing the virus was then re-suspended in DPBS. Finally, it was aliquoted and stored at -80^{o}C.

Results:

Development of selection procedure:

Upon deciding to select for switchable aptamers the first step was to decide on an appropriate method of selection. To do this, the protocol used to select aptamers for VSVΔ51 was examined.(Labib, et al., 2011) This protocol had the sole focus to obtain aptamers that bound strongly to the virus target; it was a modified cell-SELEX method altered to allow for selection of smaller targets. The main purpose of any SELEX process is to separate binding aptamers from non-binding aptamers. Through cell-SELEX the aptamers bound to the target become part of the pellet and the non-bound DNA stays in the supernatant, which could then be discarded, increasing the population of binding DNA. To select for switchable aptamers, a step was employed to release aptamers requiring Mg^{2+} and Ca^{2+} to bind. This is schematically shown in Figure 3. The selection begins by adding VSVΔ51 to the aptamer pool in DPBS and incubated for 30 minutes at room temperature. This allowed for binding between aptamers and their target to reach equilibrium, they were subsequently washed by centrifugation to remove unbound aptamers. The addition of EGTA & EDTA to remove $Ca^{2+}$and Mg^{2+} respectively allowed for the collection of aptamers that had the "switchable" functionality. These aptamers were collected and used for PCR (symmetric + asymmetric). The pool upon completion of PCR is known as the enriched pool and was used for the next round of selection. This was performed for 10 rounds and then tested to examine affinity. It was decided to continue selection for three more rounds using a method that allowed for parallel selection of aptamers using a low (500 µM), medium (2.5mM) and high concentration (10mM) of EDTA/EGTA. This yielded an additional 9 pools of aptamers which could be used for affinity analysis, one of which will be selected for cloning for specific sequences.

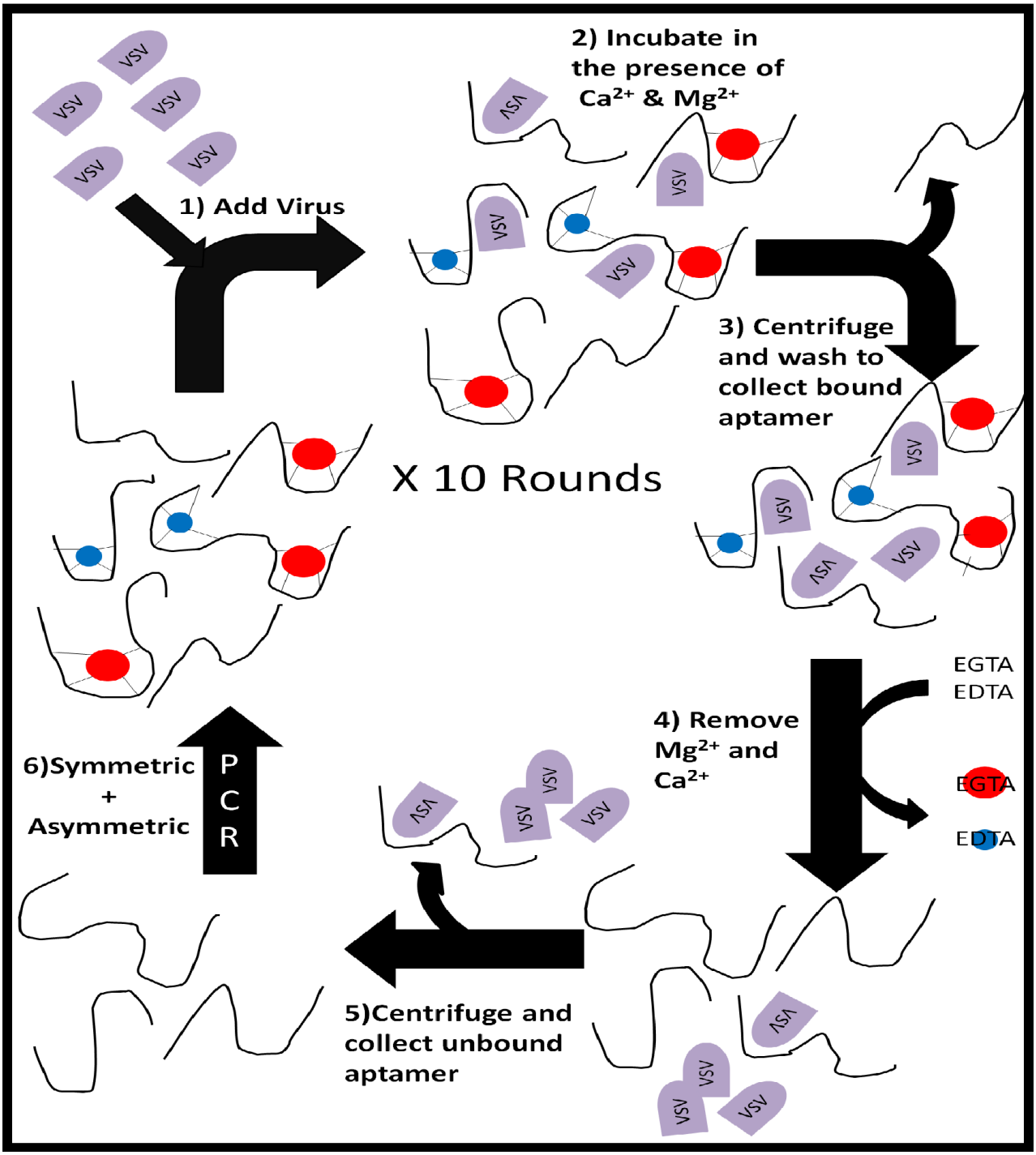

Figure 3: Switchable aptamer selection protocol through modified cell-SELEX. The process involves 6 steps: 1) add $2.5x10^9$ pfu/mL of VSV, 2) incubate aptamer with VSV in DPBS, 3) wash VSV to remove unbound aptamer, 4) add EGTA & EDTA to remove Mg^{2+} and Ca^{2+} , 5) collect unbound aptamer, 6) PCR (symmetric + asymmetric)

Aptamer affinity analysis (R1-5):

Aptamer pool affinity was analyzed using flow cytometry. Upon completion of the first 5 rounds of aptamer selection the pools' affinity was tested. Here, we solely examined the switchability of aptamer pools. (Figure 4) The purpose was to examine if the protocol was functioning correctly. We calculated the ability of the pool to release virus by using equation

$$Delta\ Switch\ =\ \%\ bound\ in\ DPBS\ -\ \%\ bound\ in\ 2.5mM\ EDTA/EGTA \quad (1)$$

The N40 library used for selection of the aptamers specific to VSV was used as a control; this showed a delta switch of approximately 20%. Rounds 1, 3 and 4 showed a delta switch of less than 10% per pool, whereas round 2 had a delta switch of approximately 35%, an increase of 15% from the library. Finally, round 5 showed the best delta switch of 53%, an increase of 33% when compared to the library. Figure 5 shows flow cytometry results in the form of histograms for library, round 1 and round 5. Here we can see two different types of switchability with respect to the library. As a control we see the N40 library bind to VSV; upon addition of EDTA/EGTA, we see a small shift back in the yellow curve. When comparing round 2 (panel b) and round 5 (panel c) we see the respective curve associated with EDTA/EGTA move back strongly with round 5 and not move at all with round 2. This is an example of a weak delta switch (round 2) and a strong delta switch (round 5). It was decided to continue with selection for an additional 5 rounds to attempt to reach 100% switchability.

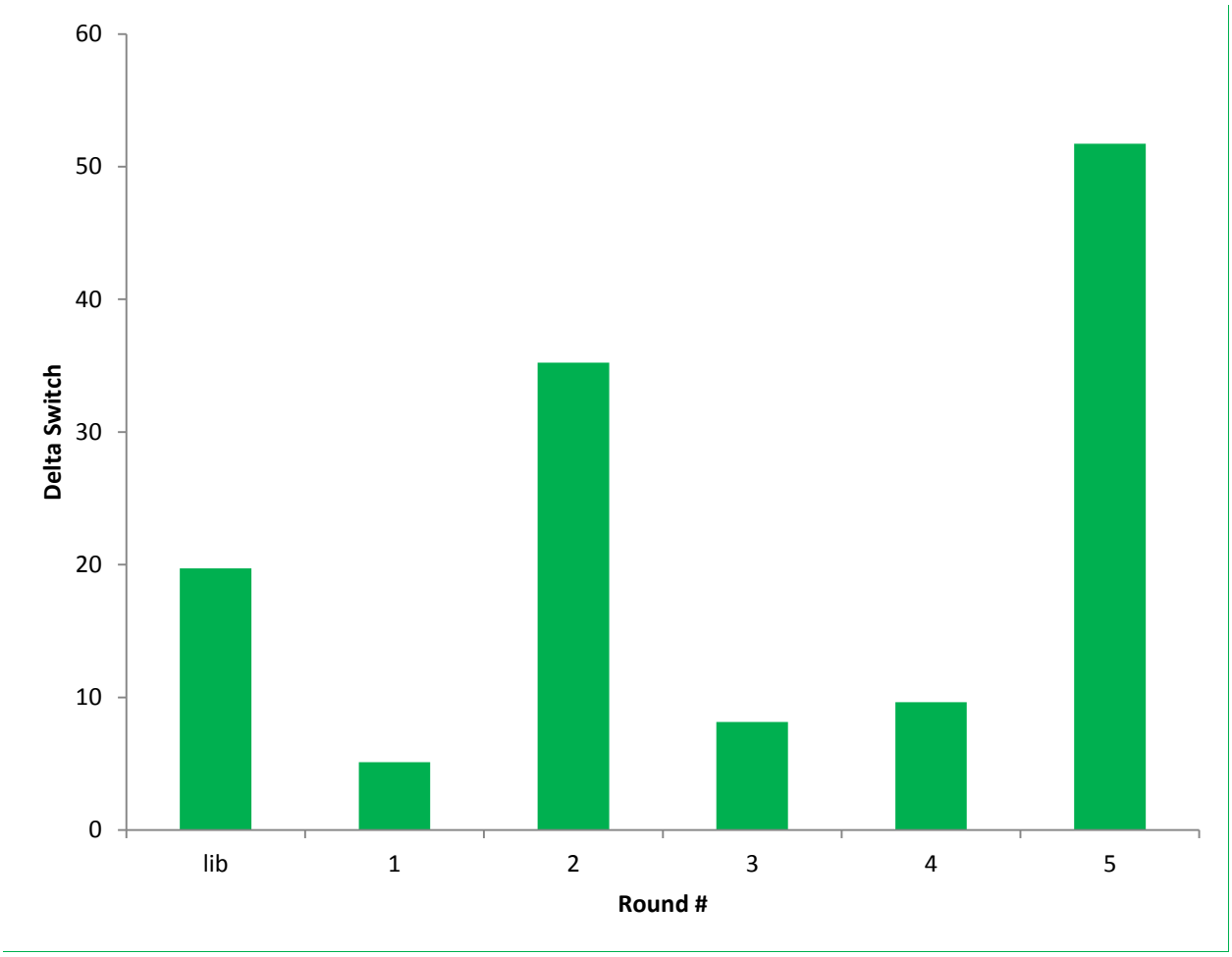

Figure 4: Measured aptamerswitchability (delta switch) for 5 rounds of selection and library. To calculate delta switch, flow cytometry analysis of binding affinities between 100 nM FAM-labeled aptamer pools and $1x10^7$ pfu/mL VSV in buffers containing DPBS or 2.5mM EDTA/EGTA. Using equation % bound in DPBS - % bound in 2.5mM EDTA/EGTA

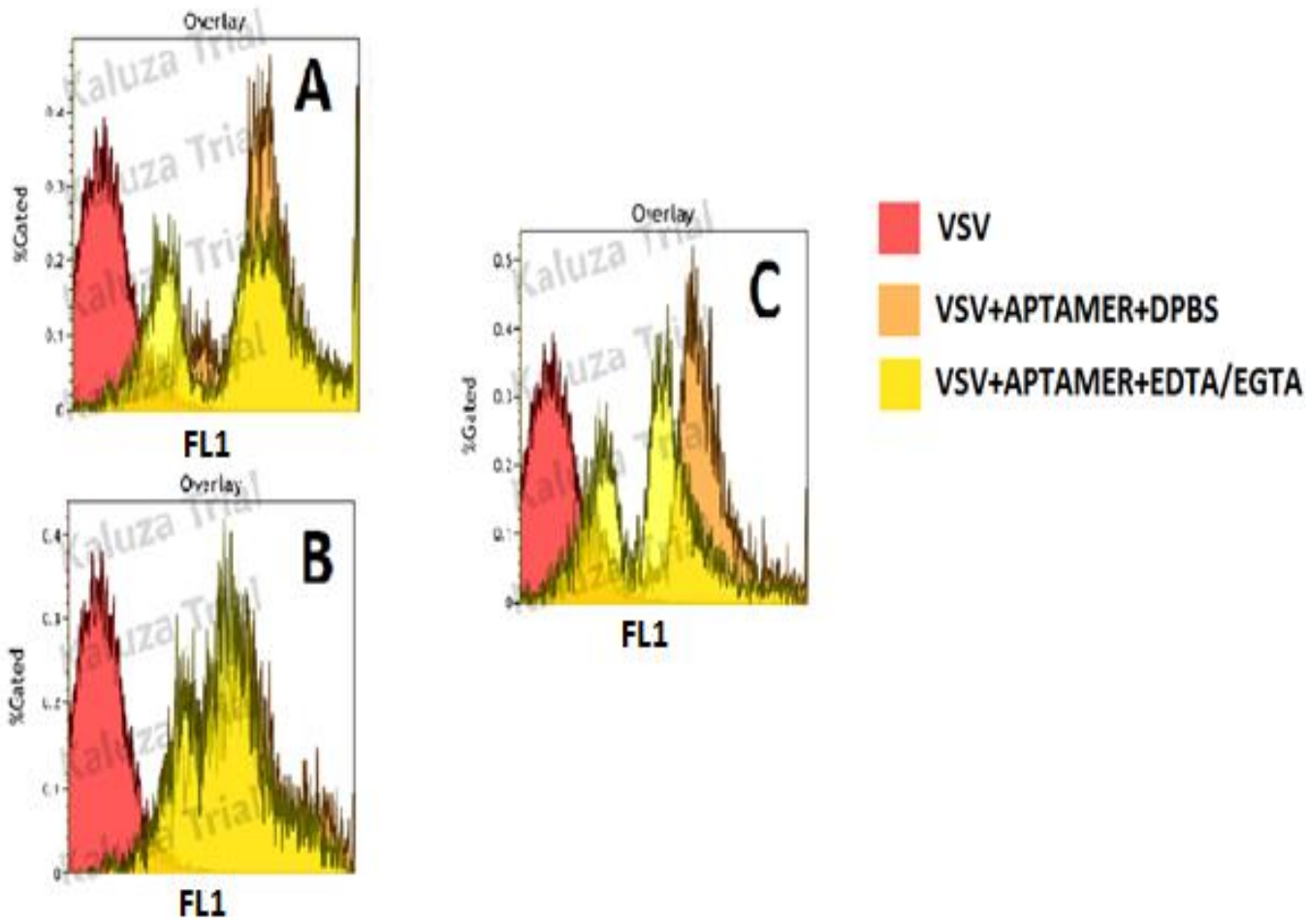

Figure 5: Flow cytometry histograms for Library, Round 1 and Round 5. Histogram compares VSVΔ51, VSVΔ51+ 100nM FAM labeled aptamer in DPBS and VSV-Δ51+100nM FAM labeled aptamer in 2.5 mM EDTA/EGTA.

Aptamer affinity analysis (R1-10)

Another 5 rounds of selection were performed upon completing affinity analysis for rounds 1-5 using two additional washing steps to increase the stringency of selection. The 10 rounds were examined for two criteria; the affinity of the aptamer pool to VSV and the delta switch for each aptamer pool. Rather than using the N40 library as a standard, it was decided to compare the aptamer pools to the initial pool given to start selection. All pools are FAM-labeled, purified and were made to a total volume of 100 µL in DPBS with 50 nM aptamer pool and 10^7 pfu/mL VSVΔ51. Figure 6 shows the binding of the 10 pools and control to VSVΔ51 for the fraction in DPBS (contains Mg^{2+} and Ca^{2+}) in blue and the fraction without (contains EDTA/EGTA) in red as percent VSV bound. Here we examine the affinity of the aptamer pools to the virus and look at pool switchability. The original aptamer pool used as a control shows very little difference in binding with the addition of EDTA/EGTA. Round three shows a large switchability upon removal of magnesium and calcium chelating ions. Figure 7 shows the delta switch calculated for each respective pool. Here we can compare how effective the aptamers can switch from their bound and unbound form. Round 0 is the lowest, followed by rounds 2 and 8, all showing a delta switch of <20%. This is a poor level of switchability as the addition of EDTA/EGTA does not cause release of VSV. A large delta switch is seen with rounds 3, 7 and 10. All the pools have a delta switch of >45%. Since pools 9 and 10 showed a good delta switch, it was decided to do three more rounds of selection to increase this functionality. To amplify selectivity, we employed a method which uses 3 different concentrations of EDTA/EGTA

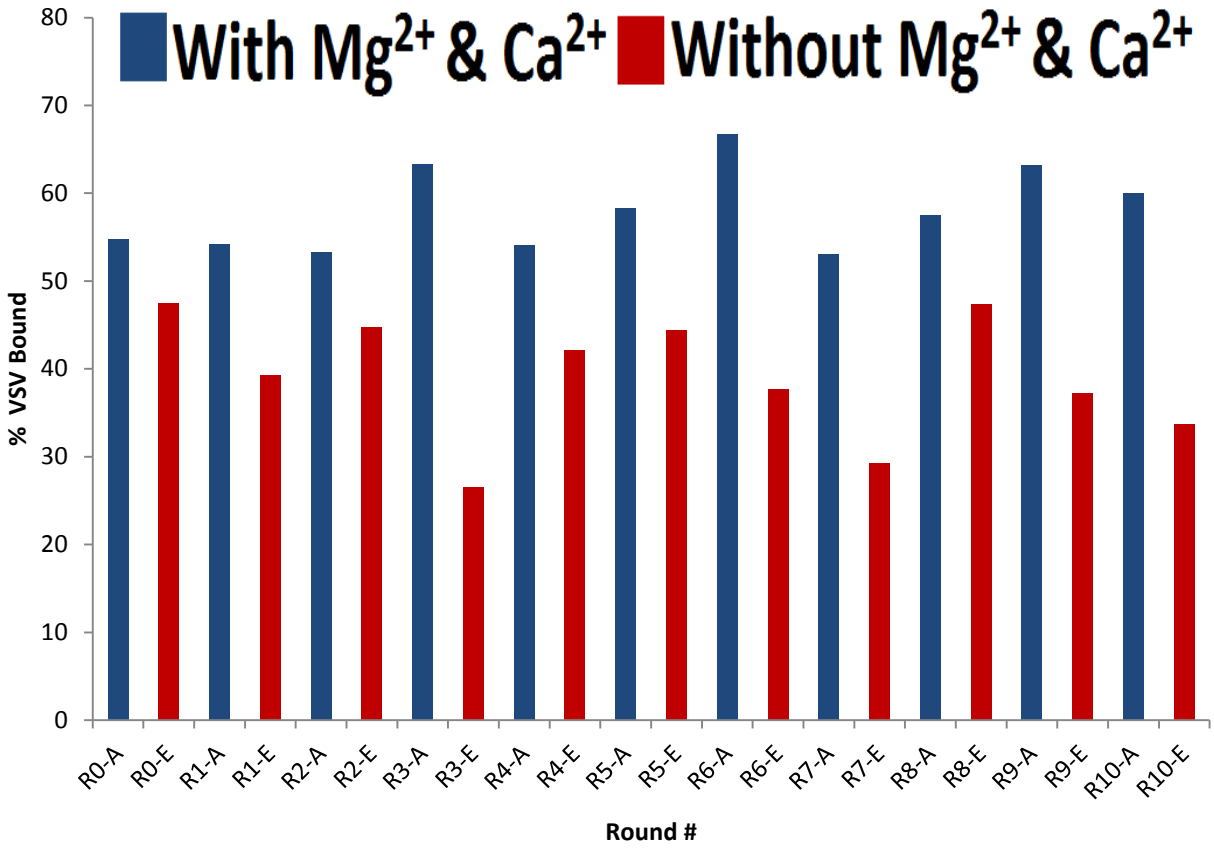

Figure 6: Binding of 10 aptamer pools and original pool to VSV. Results obtained by flow cytometry. Aptamers (50 nM) incubated with VSV (10^7 pfu) for 30 min prior to separation into 2 fractions one in DPBS ($MgCl_2$& $CaCl_2$) (blue) and one containing 2.5mM EDTA/EGTA (without $MgCl_2$& $CaCl_2$) (red).

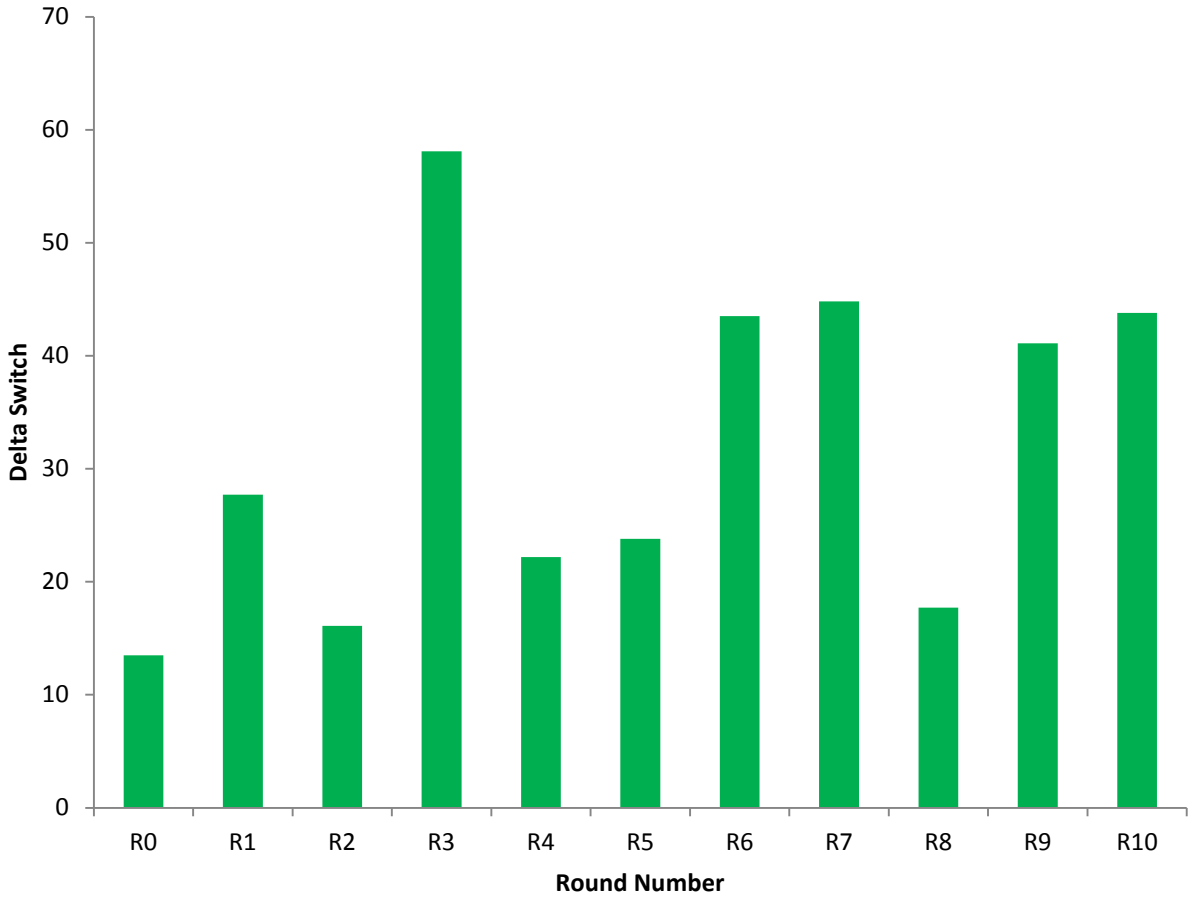

Figure 7: Delta switch for rounds 0-10 obtained using flow cytometry. 50nM aptamer pool was incubated with VSV (10^7 pfu) for 30 min prior to separation into 2 fractions. One fraction contained DPBS (Mg^{2+}& Ca^{2+}) and one contained 2.5 mM EDTA/EGTA (without Mg^{2+}& Ca^{2+}). Using equation % bound in DPBS - % bound in 2.5 mM EDTA/EGTA

and would extract aptamers that are sensitive to low, medium and high concentrations. The benefit of this method is that it would give 9 pools of aptamers after three additional rounds of selection and they would be the product of different concentrations of EDTA/EGTA.

Aptamer Affinity Analysis (R0-13) & selected rounds:

Upon completion of the three additional rounds of selection using three different concentrations of EDTA/EGTA as low (l), medium (m) and high (h), concentration pools were once again analyzed by flow cytometry. These pools used VSV that was harvested in our lab and thus the sucrose gradient purification was not used. To purify our virus we used a combination of cut-off filters and centrifugation, thus sacrificing purity to obtain a high yield. Unfortunately, when flow cytometry was done it became difficult to see the virus due to the additional contaminants. Figure 8 shows the delta switch for rounds 0-13; 3 pools show exceptionally low delta switch. Rounds 8, 12 (h) and 13 (l) show very low sensitivity to EDTA/EGTA. In addition, rounds 2, 3, 10, 11 (h) and 13(h) show a good delta switch. We then used TOTO-3 dye to locate VSV on the dot plot on flow cytometry system as the dyed virus can be seen in the FL4 (emission 675nm ± 25) filter. (Figure 9) After optimizing the gating strategy, flow cytometry was performed again using selected pools which have shown good binding and switchability. Rounds 0, 2, 3, 10, 11h and 13h were selected for further analysis and were examined for delta switch and percent aptamer released. Delta switch for 6 rounds can be seen in figure 10; here we can see that round 3 has the best delta switch followed by round 10. The second parameter examined is percent aptamer released; this was calculated by:

$$\%\ aptamer\ released = 100\% - \frac{(\%\ bound\ after\ EDTA{+}EGTA)(100\%)}{\%\ Bound\ in\ DPBS}. \quad (2)$$

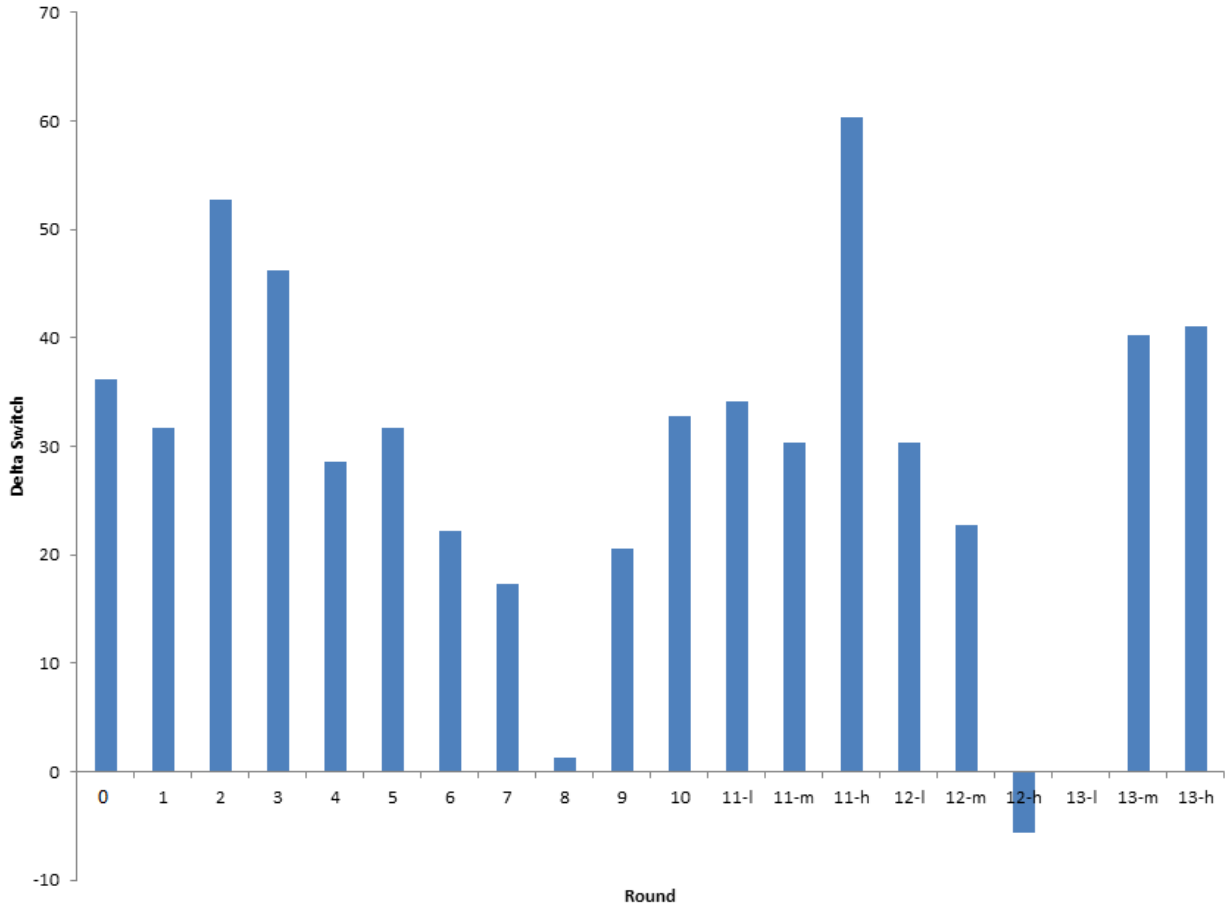

Figure 8: Delta switch for rounds 0-13 obtained using flow cytometry. 50nM aptamer pool was incubated with VSV (10^7 pfu) for 30 min prior to separation into 2 fractions. One fraction contained DPBS (Mg^{2+}& Ca^{2+}) and one contained 2.5 mM EDTA/EGTA (without Mg^{2+}& Ca^{2+}). Rounds 11-13 have three fractions each indicating low (l), medium (m) and high (h) concentration EDTA/EGTA was used in selection. Using equation % bound in DPBS - % bound in 2.5 mM EDTA/EGTA

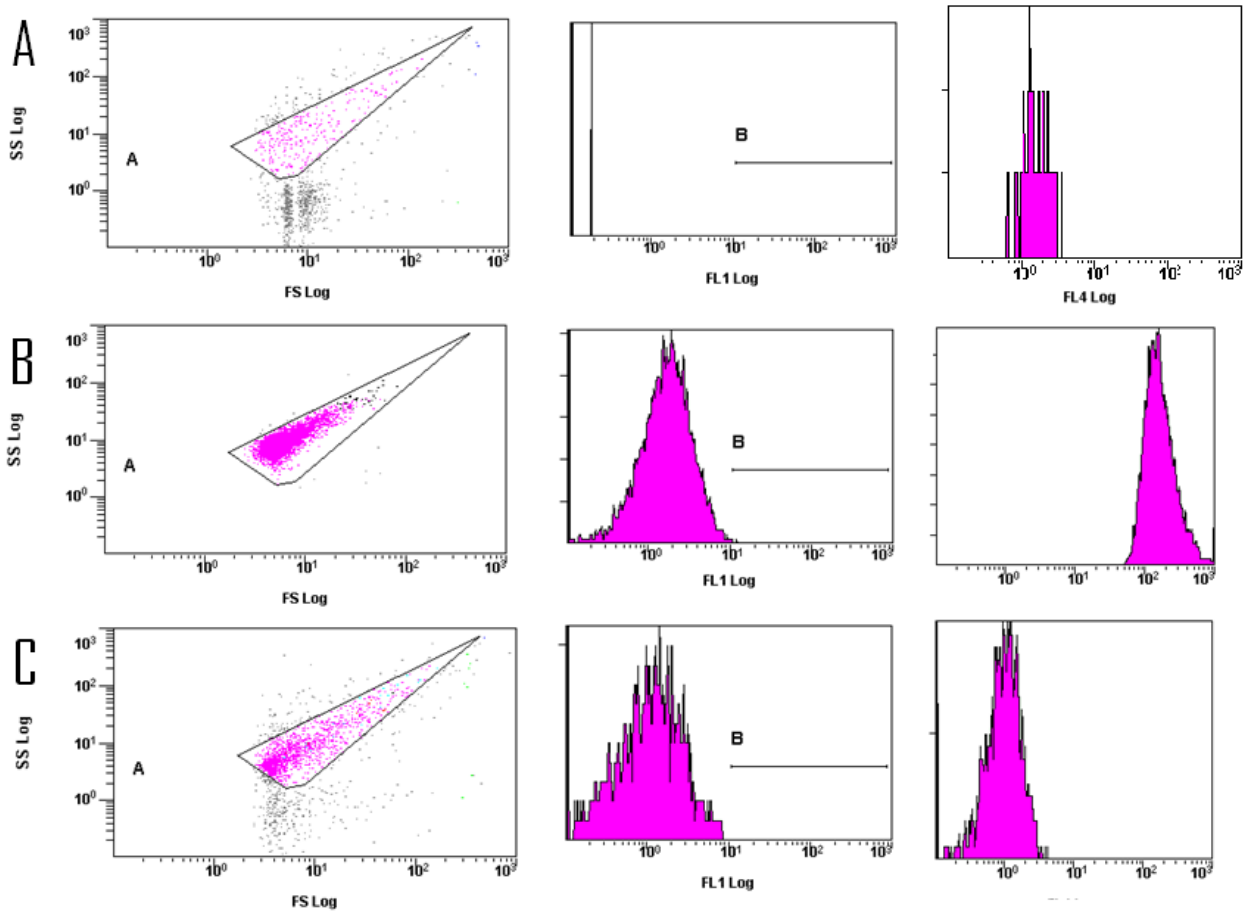

Figure 9: Gating for forward scattering (FS) / side scattering (SS), FL1 detector (emission 525nm ±25) and FL4 detector (emission 675nm ± 25). Data obtained using Beckman FC500 series flow cytometer. (A) DPBS filtered through 0.2 µM cut-off filter. (B) VSVΔ51 + TOTO-3 pre-incubated for 30 min at 25°C. (C) VSVΔ51

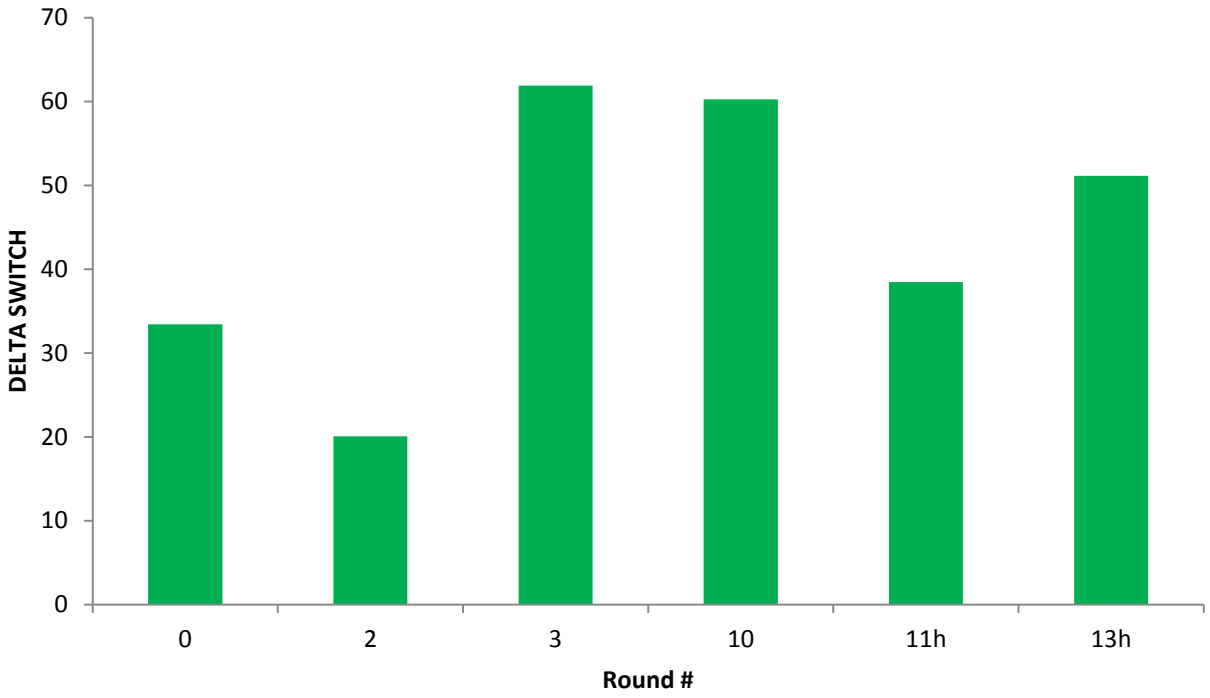

Figure 10: Delta switch for rounds 0, 2, 3, 10, 11h, 13h obtained using flow cytometry. 50 nM aptamer pool was incubated with VSV (10^7 pfu) for 30 min prior to separation into 2 fractions. One fraction contained DPBS (Mg^{2+}& Ca^{2+}) and one contained 2.5mM EDTA/EGTA (without Mg^{2+}& Ca^{2+}). For rounds 11 and 13 "h" indicates a high concentration (10mM) EDTA/EGTA was used during selection. Using equation % bound in DPBS - % bound in 2.5 mM EDTA/EGTA

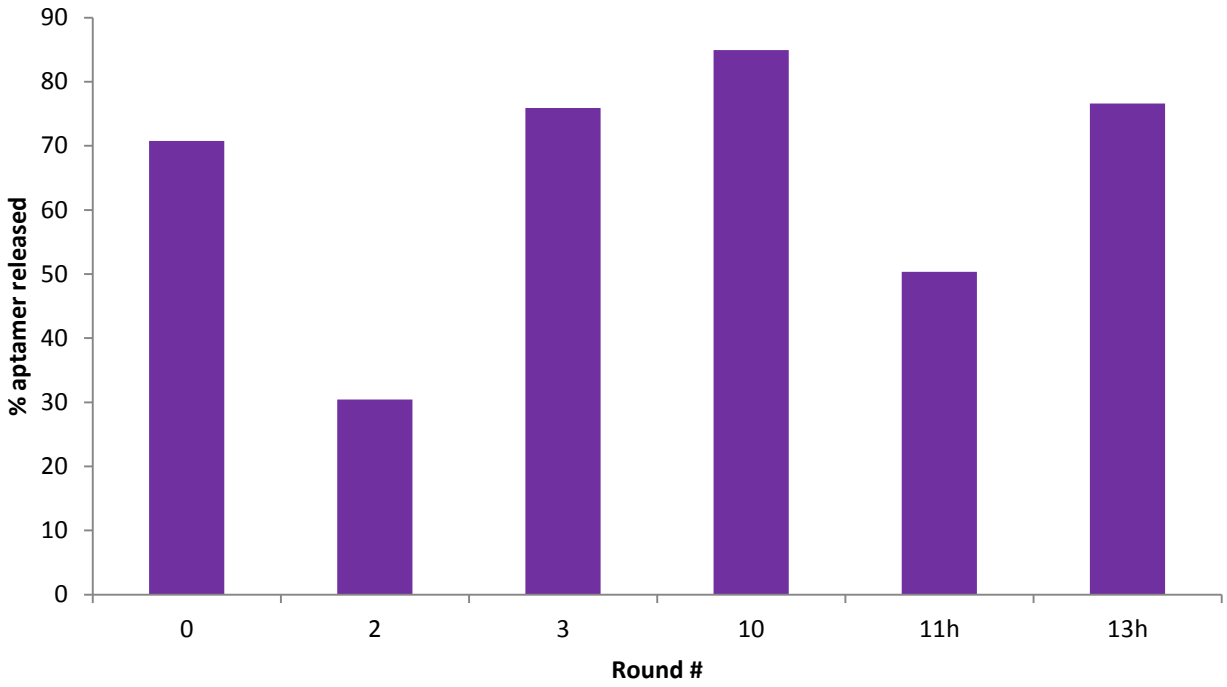

Figure 11: Percent of aptamer released upon addition of EDTA/EGTA for rounds 0, 2, 3, 10, 11h, 13h obtained using flow cytometry. 50nM aptamer pool was incubated with VSV (10^7 pfu) for 30 min prior to separation into 2 fractions. One fraction contained DPBS (Mg^{2+}& Ca^{2+}) and one contained 2.5 mM EDTA/EGTA (without Mg^{2+}& Ca^{2+}). For rounds 11 and 13 "h" indicates a high concentration (10mM) EDTA/EGTA was used during selection.

Figure 11 shows that aptamer pool 10 gives the most released aptamer upon addition of EDTA/EGTA. This indirectly indicates the amount of VSV liberated with the addition of EDTA/EGTA and therefore gives information on the purification potential of the pool. Round 10 shows comparable delta switch with round 3 but a 10% increase in aptamer release. Figure 12 shows the overlay histogram for round 10, here we can see a shift to the right from VSV to VSV+aptamer in DPBS. We then see the shift to the left from VSV+aptamer in DPBS to VSV+aptamer with EDTA/EGTA. Thus, round 10 was selected for cloning.

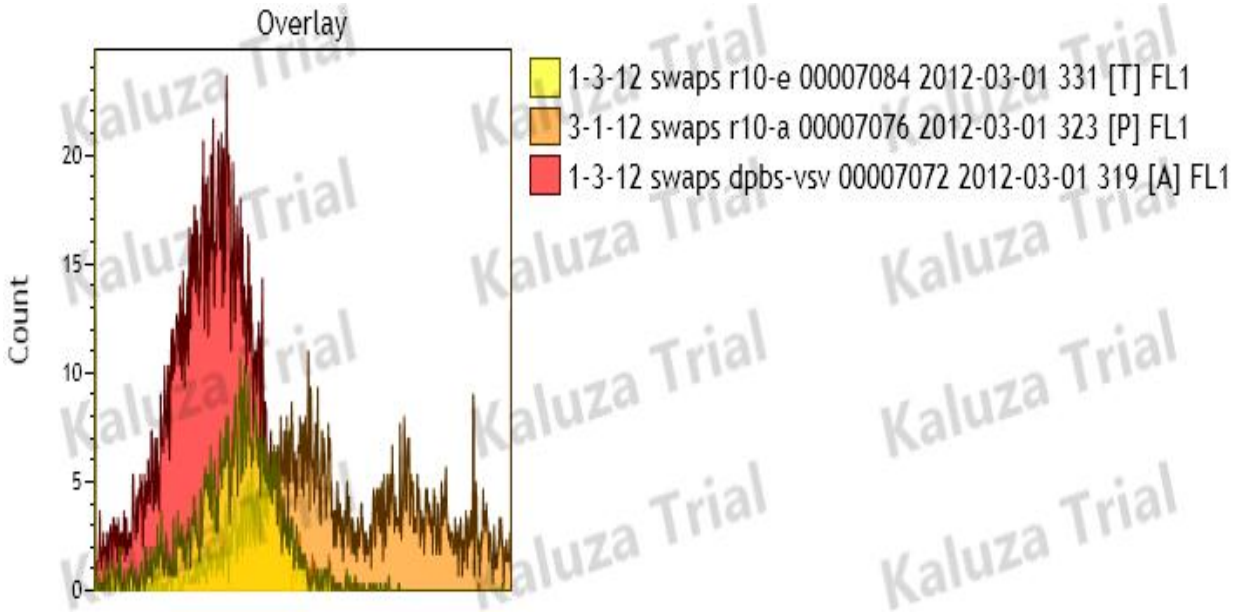

Figure 12: Flow cytometry histograms for round 10. Histogram compares VSV in DPBS, VSV+ aptamer pool 10 in DBPS (a) and VSV+aptamer pool 10 in PBS with (EDTA+ EGTA).

Discussion:

Aptamer Selection:

It is a widely known principle in aptamer selection that divalent cations aide in the stabilization of aptamer tertiary structures.(Hart, 2000)(Nomura, et al., 2010) The two most commonly employed cations in aptamer selection are magnesium and calcium; they can be found in DPBS at 0.493 mM and 0.901 mM respectively. Here we attempt to exploit the use of these cations through a modified cell-SELEX to create switchable aptamers (SwAps).These aptamers will have a switchable functionality allowing them to bind to their targets in the presence of Mg^{2+} and Ca^{2+} ions and have the ability to release their targets once the ions are removed. To allow for this functionality, an additional step in the modified cell-SELEX process was added using 2.5 mM EDTA and EGTA in PBS. A total of 13 rounds of selection were performed from a pool already selected to bind VSVΔ51.

Normally, there are two features that are important when selecting for aptamers, affinity and selectivity. These were already accounted for in the selection from which our pool originated. Thus, our purpose was to add switchable functionality that can be used to aide in VSVΔ51 purification. The first 5 rounds were performed using three washing steps; this was increased in the subsequent rounds to increase stringency in selectivity. These steps are imperative for removing all unbound and weakly bound aptamers. These non-specific binding aptamers are known as being “sticky” as they have no specificity or selectivity. (Shangguan, et al., 2006) Upon completion of washing steps, we incubated the virus pellet with EDTA/EGTA for 30 minutes. This allowed for these chelating agents to sequester the divalent cations. The expected result would be a conformational change in those aptamers requiring Mg^{2+} and Ca^{2+} for

binding. Therefore, upon centrifugation, aptamers that require cations for binding will be in the supernatant while the bound ones will be with the virus pellet. Rounds 11-13 were performed using three different concentrations of EDTA/EGTA: low (500 µM), medium (2.5 mM) and high (10 mM). This is employed as a means to separate aptamer pools that are sensitive to different concentrations of EDTA/EGTA. It has been previously reported that a decrease in aptamer pool binding can occur from performing too many rounds of selection. (Cox & Ellington, 2001) Although the mechanism is unknown, this phenomenon can be compensated for by doing the aforementioned scheme at different concentrations of EDTA/EGTA as it yields several varying pools in a few rounds.

Affinity Analysis

Aptamer affinity analysis plays an important role in all selection processes, as before cloning an aptamer pool one must know which has the greatest affinity to the target. In the case of switchable aptamers, one must find which pool offers strong switchability without sacrificing the respective affinity to the target. The method of choice for analyzing the data was flow cytometry. During analysis, the original stock of VSVΔ51 that was purified using sucrose gradient was depleted, thus more was harvested using Vero cells. Since, the purpose for VSVΔ51 here was strictly analysis; the sucrose gradient purification was not performed as it was deemed unnecessary. Flow cytometry analysis showed a high level of contamination, this was confirmed independently by capillary electrophoresis (data not shown). It was decided to stain the virus with TOTO-3 dye (Invitrogen) so as to allow identification of the virus on the FL4 filter (emission, 675 ±25nm). (Johnson & Spence, 2010) This would allow for the tracking of the dyed virus in a separate channel so as to not interfere with FL1 which is used to examine the FAM-labeled aptamer-VSV binding. Another problem occurring with the flow cytometry system used

is caused by the size of the virus. VSV is approximately 70 x 200 nm, which is very small in comparison to the size of a cell for which flow cytometry is typically used. (Hercher, Mueller, & Shapiro, 1979)

The use of FAM-labelled aptamers allowed for easy detection of aptamer-VSV binding. Small molecules, such as aptamers, are too small to be detected by flow cytometry, which is beneficial as it does not change the size of the virus (no need to re-gate) and it allows one to examine binding of fluorescent aptamers. Once the gate has been set up, one can examine FL1 channel (emission of 525±25 nm) to examine binding. Unbound virus has low fluorescence and thus when aptamers specific to VSV are added, they bind, thus causing a shift in the FL1 channel due to increased fluorescence. It should be noted that some particles may auto-fluoresce but this is corrected by compensation and was not the case here. The shift is proportional to the amount of binding aptamers that are present and thus different pools can be compared using this method.

Since the method of selection discussed is to find switchable aptamer pools additional analysis of flow cytometry data must be made. We developed the term delta switch to relate the difference in binding between aptamer and VSV with magnesium and calcium and those without these chelating ions. What this means with respect to FL1 histograms is that when binding occurs in the presence of Mg^{2+} and Ca^{2+}, a shift to the right is seen (If the aptamers selected for the target are binding.). When EDTA/EGTA is added they sequester the cations and the expected result would be a back-shift to the left. Thus, unlike normal aptamer selection when binding is the sole factor of interest, here we identified aptamer pools that bind strongly to VSV but that also show this "switchable" functionality when Mg^{2+} and Ca^{2+} are removed.

The first five rounds of selection were performed after obtaining pool 0. We examined one element for the aptamer pools which were the respective delta switch values using the N40 library as a control. The delta switch for the N40 library was found to be greater than some of the pools. This is likely the result of non-specific binding, which is a common problem with aptamer selection. This is usually remedied by using masking DNA/RNA, such as yeast RNA, to block these non-specific points of binding. (Shi, Fan, Sevilimedu, & Lis, 2006) It should be noted that the DNA library does not show the same high affinity to VSV as the other pools which have been selected for the target. (Labib, et al., 2011) This is important to note that although delta switch gives information on switchability, strong selective binding to VSV is the prerequisite for a SwAps pool. The two pools that showed a good delta switch in comparison to the N40 library were pools 2 and 5.

Selection was continued for another five rounds and the pools were again analyzed. We decided to use the initial pool (round 0) as a control instead of the N40 library as round 0 shares a similar affinity to aptamers for VSV and in this case it could be considered as a pseudo-library. The delta switch was small, which indicates binding of the pool to VSV is largely unaffected by the presence or absence of magnesium and calcium. In contrast, round three showed a very good delta switch and was also one of the best binding aptamer pools. Rounds 7 and 10 were also promising pools which showed good binding and a high delta switch, whereas rounds 1, 2 and 8 did not show good switchability. Since rounds 1 and 2 were at the beginning of selection, it was expected that they would not show the expected switchable functionality. One would think that as the number of rounds of selection increases, the binding and switchability character would also increase linearly. This is seldom the case in aptamer selection as after every round of selection we introduce mutations during PCR which may be beneficial or detrimental to binding.

Although, we did see some pools with the desired switchability character, it was decided to continue selection to see if we had reached a plateau in switchability. It was decided that we would continue with selection to attempt to obtain a better pool with more switchable character.

We decided to employ a technique using three different concentrations of EDTA/EGTA which would result in more additional pools in less rounds of selection. These pools were also analyzed by flow and it was found that rounds 11 and 13, both selected with high concentrations of EDTA/EGTA, as well as rounds 2 and 3 showed good switchability. We calculated % released aptamer to measure how much of the VSV bound we can recollect by adding EDTA/EGTA. Round 10 showed the best % released aptamer as well as a good delta switch and had been chosen for cloning.

For future work, we must complete the cloning, currently in progress, and measure the respective affinity and delta switch of each individual aptamer sequence. This will also include calculating the dissociation constant (K_d) for the selected sequences and aptamer pool. Since the main objective of these aptamers is to purify VSVΔ51, we plan to examine their capabilities in a bioassay. Here we will bind the aptamers to beads and mix them with unpurified VSV in order to attempt to obtain the release of pure virus. The samples will be analyzed by two methods, flow cytometry and viral quantitative capillary electrophoresis (viral QCE). Finally, we would like to examine the effects of temperature (Jayasena, 1999) and pH (Hianik, et al. 2007) on aptamer binding. The principle being that upon addition of EDTA/EGTA we could implement a change in temperature or pH to further decrease aptamer binding to increase the yield of virus obtained.

Conclusion:

Of the discussed purification methods, the sucrose gradient is the most commonly employed; unfortunately this method has been found to decrease infectivity by lowering glycoproteins from the virus envelope. Here we identify a novel method of selecting aptamers and offer an alternative method to purifying VSV. The use of aptamers is increasing as they offer high affinity and selectivity towards their respective targets. The method we offer here adds a new level of functionality to an already increasingly popular class of biomolecules. Furthermore, this functionality can potentially be added to existing aptamers by performing additional rounds of selection using the method described here. Viral therapeutics are an emerging class of medicine; for this to be feasible the OVs must be free from contamination prior to injection. Using SwAps we offer a method of affinity purification which we expect to not alter the infectivity of the virus and which will yield pure product. From the results obtained, it is clear that the next step is to clone pool 10 and test clones for their switchability and purification capabilities.

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