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14. ABSTRACT

The transmissible spongiform encephalopathies (TSE) chronic wasting disease (CWD) of elk and deer has the potential for transmission to human beings. Reliable antemortem diagnostic tests for CWD are necessary for its control. Development of a novel diagnostic probe termed aptamers to detect CWD is proposed. Aptamer selections against 1) tyrosyl-tyrosyl-arginine (YYR) tripeptide thought to be exposed in PrP^{Sc} but not in PrP^{C} and 2) CWD PrP^{Sc} using a novel electrodialysis SELEX technique were assessed by 1) reduction in aptamer pool complexity by cloning and sequence analysis and 2) direct target binding assays. Neither of these selections yielded aptamers specific for PrP^{Sc}. To overcome the difficulties of removing potentially useful aptamers to CWD PrP^{SC} during the negative selection phase, a novel antibody displaying selected motifs of PrP^{SC} that have been shown to be involved in prion protein mis-folding will be used as our target for aptamer selection. Two such motif grafted antibodies for PrP sequences 89-112 and 136-158 were developed by Williams laboratory at the Scripps Research Institute and have graciously been provided to us through an MTA. Experiments are underway to select aptamers to the PrP^{SC} specific motifs displayed on these antibodies. A capillary electrophoresis aptamer selection protocol is being developed for the selection process.

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No subject terms provided.

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Introduction

Transmissible spongiform encephalopathies (TSE) such as mad cow disease and its human equivalent variant Creutzfeldt-Jakob disease are transmitted by ingestion of meat contaminated with infective prion protein (PrP^{sc}). The incubation period for TSEs is many months to years such that it is difficult to identify the source of infection and initiate effective control measures. Recently, a TSE of elk and deer termed chronic wasting disease (CWD) has raised concerns that it too may be transmissible to human beings via ingestion of contaminated meat. Reliable antemortem diagnostic tests for TSEs are necessary for control of these diseases. Because TSEs result from mis-folding of endogenous normal prion protein (PrP^{c}) to form protease resistant PrP^{sc} isoform, diagnostic tests need to sensitively detect PrP^{sc} in samples and specifically distinguish PrP^{sc} from PrP^{c}. Monoclonal antibodies (Mab) have not been produced which are both sensitive and specific for PrP^{sc}. Commercial Mab-based immunoassays are available for use in detecting PrP^{sc} in biopsy or postmortem histologic or homogenized brain tissue samples, but these require removal of PrP^{c} by proteinase K digestion. Therefore, a rapid, specific method of direct antemortem identification of PrP^{sc} is needed. At the time that this project was proposed, novel diagnostic probes termed aptamers have been developed to PrP^{c}, however, these aptamers did not distinguish between PrP^{c} and PrP^{sc} [1]. Aptamers have an advantage over Mabs because aptamers can be engineered to signal the results of a diagnostic test directly, whereas Mab require extensive protocols to produce results. These engineered aptamers are called aptamer beacons. We are developing aptamer beacons as molecular switches to turn “on” a novel diagnostic technology termed amplifying fluorescing polymer (AFP) when an infectious agent is present. AFP is 1,000 times more sensitive than currently available diagnostic technologies. The proposed project will develop aptamer beacons to CWD for subsequent use in developing an AFP-based antemortem diagnostic test for CWD.

Body

Aptamer selection (systematic evolution of ligands by exponential enrichment or SELEX) is an iterative process of binding a pool of random sequence oligonucleotides (aptamer candidates) to the desired target, partitioning of the bound species from the unbound species, and polymerase chain reaction (PCR) copying of the bound species followed by repetition of the process until only a few oligonucleotide species that exhibit high specificity and affinity for the target remain in the pool at high copy number. To overcome the negative result of Proske et al (2002) with respect to selection of aptamers that specifically recognize PrP^{sc}, we originally proposed 1) to direct aptamer selection against all linear epitopes for aa 91 to 245 of deer PrP so that pools of aptamers to all linear epitopes of $PrP_{91\text{-}245}$ are available for subsequent crossover SELEX, and 2) to use a crossover SELEX approach [2] to positively select for aptamers that recognize PrP^{sc} but to do recognize PrP^{c}.

Target	Selection Strategy	Advantages	Alternative Selection Strategy
Peptide library	Positive selection of aptamers to cover all linear epitopes of PrP_{91-245}	Yield aptamers for all linear epitopes of PrP_{91-245} so that potentially distinguish-ing epitopes are not missed	Positive selection of the aptamer pool against the YYR motif thought to selectively displayed in PrP^{SC}
Crude CWD PrP^{sc}	Positive selection of aptamers to all targets in crude CWD PrP^{sc} preparation	Removes aptamers from pools that do not recognize corresponding epitopes in PrP^{sc}	Same as proposed using novel electrodialytic SELEX against CWD PrP^{SC}
Purified recombinant bovine PrP^{c}	Negative selection of aptamer to PrP^{c}	Removes aptamers from pools which also recognize PrP^{c}	Same as proposed, but using elk recombinant PrP^{C} rather than recombinant bovine PrP^{C}

Since we proposed the strategy outlined above, two research groups have reported findings that bear on our experimental approach. First, a group at Oxford selected 2'-fluoro-RNA aptamers that exhibit 10-fold higher affinity for bovine PrP^{sc} than for PrP^{c} [3]. These researchers demonstrated the feasibility of selection of aptamers that can recognize PrP^{sc} preferentially over PrP^{c} by targeting epitopes that are more specific for PrP^{sc} than for PrP^{c} [4]. The other group identified tyrosinyl-tyrosinyl-arginine (YYR) as the binding epitope for a monoclonal antibody that specifically recognizes PrP^{sc} from human beings, cattle, sheep, and mice [5]. These authors speculate that YYR is hidden in the interior of the normally folded PrP^{c}, but surface exposed in PrP^{sc}. Based on this finding we altered our peptide library initial enrichment SELEX to select against immobilized YYR. No further data has been published on the applications of this monoclonal antibody, leading some colleagues to speculate that this epitope may not be specific for PrP. As shown in the right hand column of the table above, our selection strategy has changed to take into account these developments and others such as the superiority of electro-separation of unbound from target bound aptamers for SELEX and the availability of a plasmid for production of recombinant elk PrP^{C}.

The **Statement of Work** included the Objectives 1 and 2 for Year 2:

Objective 1 Select aptamers for CWD abnormal prion (PrP^{sc}) (Months 1-15)

Tasks 1.1 Develop protocol for peptide library SELEX (Months 1-3)

1.2 Conduct SELEX with peptide library array for deer prion (PrP_{91-245}) (Months 3-9)

1.3 Prepare and assess preparation of crude cervid PrP^{sc} (Months 4-9)

1.4 Conduct SELEX for crude cervid PrP^{sc} (Months 9-12)

1.5 Conduct negative SELEX for bovine recombinant normal prion ($rPrP^{C}$) (Months 12-15)
1.6 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

Deliverable: Reduced aptamer pools selected for sensitive and specific recognition of PrP^{sc}

Objective 2 Sequence selected aptamers species and down-select for aptamer beacon engineering (Months 15-24)
Tasks 2.1 Clone reduced aptamer pools from Objective 1.5 (Months 15-18)
2.2 Sequence aptamer clones (Months 18-21)
2.3 Assess sequence homologies from clones for family relationships (Month 21)
2.4 Determination of sensitivity and specificity of aptamers representative of aptamer families from 2.3 for CWD prion (PrP^{sc}) (Months 21-24)
2.5 Ranking of sensitivity and specificity of aptamer families for CWD prion (PrP^{sc}) (Month 24)
Deliverable: Aptamer sequences sensitive and specific recognition of PrP^{sc}

Progress on Objectives

1.1 Develop protocol for peptide library SELEX (Months 1-3)

The initial SELEX targeting strategy was modified based on a July 2003 paper describing a differential epitope that enabled antibodies generated against this epitope to discriminate the normal and misfolded forms of bovine PrP [5]. This epitope is a tandem YYR motif which is common to all species of PrP is located at residues 152-154 and 165-167 for the elk prion protein. We altered our targeting strategy to direct the aptamer pool SELEX towards recognition of this motif on elk PrP^{sc}. This peptide provides an excellent target for aptamer binding because of the positive charge on the arginine and opportunities for hydrogen bonding on the tyrosine residues. A suitable immobilized YYR peptide SELEX target was prepared using a SPOTs kit (Sigma Genosys) using an N-terminal glycine to immobilized the YYR peptide on a cellulose membrane.

1.2 Conduct SELEX with peptide library array for deer prion (PrP_{91-245}) (Months 3-9)

Figure 1 shows a schematic of the aptamer cassette used for SELEX, and Figure 2 shows a scheme of the SELEX process used. Amplifying short oligonucleotides such as the aptamer cassette used herein can be difficult and can result in the production of high molecular weight (HMW) ‘parasites’. These HMW parasites probably result from annealing of primers to internal stretches of the random insert of the aptamer cassette and subsequent amplification. We optimized a protocol for reproducibly amplifying our aptamer cassette while minimizing the development of these parasites. This resulted in the two step PCR cycling parameters shown at Figure 3. Note that the extension step

in most PCR reactions is omitted and that the annealing temperature is relatively high. The HMW parasites are shown in the gel on the right in Figure 4 for the three-step PCR protocol. The correct 73-base aptamer is aligned with the third molecular weight marker from the bottom. The gel on the left hand side of Figure 4 is for the two-step PCR, and no HMW parasites are produced.

The GYYR peptide immobilized on cellulose membranes via the N-terminal glycine served as our initial target for aptamer selection. The SELEX protocol we developed specifically for the cellulose immobilized peptide used negative selection against non-derivatized cellulose followed by positive selection for aptamers that bound to the GYYR derivatized cellulose. A two step PCR protocol was used to amplify bound aptamers. Four rounds of SELEX were completed using this protocol. We anticipated using six to eight rounds of SELEX against the YYR target prior to cloning the reduced aptamer pool and assessment of specificity and affinity for the YYR target. However, additional rounds of SELEX with this target above four were not possible due to an unknown PCR inhibitory effect, and sequencing of this pool showed no reduction in heterogeneity. Some uncertainty exists about whether the YYR target is the best target for initial selection against cervid PrP^{SC}. Some of our colleagues expressed concern that this epitope may be non-specific for PrP^{SC} because it is a commonly occurring protein motif. With the combined result of no apparent selection pressure and doubts about the specificity of target, this approach was abandoned in favor of electrodialysis with enriched cervid PrP^{SC}.

1.3 Prepare and assess preparation of crude cervid PrP^{SC} (Months 4-9)

We have successfully optimized and performed two methods of preparing cervid PrP^{SC} that have been developed by others: 1) a precipitation with phosphotungstate (PTA precipitation) method and 2) two ultracentrifugation methods. Each has advantages and disadvantages.

The PTA precipitation involves subjecting a 10% w/v homogenate of crude tissue to brief centrifugation to remove cellular debris. The supernatant is then digested with proteinase K which essentially removes PrP^{C} from the preparation, but also truncates the amino terminal 90 amino acids of PrP^{SC}. The PrP^{SC} is then precipitated with phosphotungstate in the presence of mild detergents. This contaminates the preparation with high amounts of salts which are difficult to remove. This results in a degraded form of PrP^{SC}, but it is essentially free of PrP^{C}. Figure 5 shows the results of a serial ten-fold dilution of this preparation run on a 15% polyacrylamide gel in the presence of SDS, blotted to nitrocellulose, probed with Mab 6H4, using an alkaline phosphatase conjugated secondary antibody and developed with BCIP/NBT. As with the other preps, the multiple banding pattern represents the various glycoforms of the protein.

The ultracentrifugation method is more technically demanding and may not completely remove PrP^{C} from the preparation, but results in a very high enrichment for full length PrP^{SC} without contaminating salts. Briefly, a 10% w/v crude brain homogenate is

subjected to brief ultracentrifugation to remove cellular debris. The supernatant is then subjected to a further round of ultracentrifugation with or without prior digestion with proteinase K. The pellet is then resuspended by sonication for use as target PrP^{SC}. Figure 6 represents a Western blot of Beeke's preparation which involves shorter ultracentrifugation steps but does use a proteinase K digestion. This blot was prepared similarly to the PTA prep except it is developed using an HRP conjugated secondary antibody and chemiluminescent substrate (ECL from Pierce Biotechnology Inc.). Figure 7 shows a similarly prepared Western blot of Bolton's preparation in which much longer ultracentrifugation steps but does not subject the sample to digestion with proteinase K. Figure 8 shows a Western blot of a serial dilution of crude brain homogenate and demonstrates the sensitivity of the primary antibody 6H4. Figure 9 shows a comparative Western blot using different primary antibodies to test their utility. Figure 10 shows denaturing polyacrylamide gels stained with SYPRO Ruby protein stain to demonstrate relative degree of purity from each preparation.

Dr. Blair has worked with two nationally known prion laboratories in developing these protocols for use in aptamer selection. He traveled to the laboratory of Dr. Katherine O'Rourke at the USDA lab at Washington State University to perform the PTA precipitation. He has also traveled to Creighton University to Dr. Jason Bartz's laboratory to learn two ultracentrifugation methods. We now have stocks of highly pure cervid PrP^{SC} from all preparative methods to use in SELEX.

1.4 Conduct SELEX for crude cervid PrP^{sc} (Months 9-12)

The ideal method of target presentation for aptamer selection is a free, unbound target in solution. This allows optimal interaction of the aptamer with all potential binding interfaces without the potential steric hindrance introduced by an immobilization strategy. It also obviates the need for negative selection against the immobilization media. The primary drawback to such a presentation strategy is recovery of binding species. One group approached this problem by using capillary electrophoresis to separate bound from unbound species and were able to select binding aptamers within four rounds of selection (Mendonsa and Bowser 2004). We have developed a novel electrophoretic separation strategy using dialysis membranes to trap a relatively large protein target within an electric field after interaction with a pool of aptamers. After a period of electrophoresis, the unbound oligonucleotides are driven out of the membrane, leaving bound species and the protein target within the membrane. Aliquots of this electrodialysed solution inside the dialysis membrane are then used for PCR amplification of binding species.

Application of our electrodialytic SELEX to PrP^{SC} was halted in September 2004 by Oklahoma State University Institutional Biosafety Committee (OSU-IBC), principally over concerns on disposal of prion wastes. Our project is the first prion project at OSU, and although our protocols were originally approved, the OSU-IBC felt that they needed to reconsider all of our prion protocols. Upon review of our protocols, they were

concerned that prion wastes should be both treated with prion inactivating agents such as clorox or NaOH and incinerated. We agreed to these changes; however, the regulatory process required four months to be completed. While our work with prions was stopped, we continued SELEX method development with an alternative protein target.

A total of fourteen rounds of SELEX were performed:

ROUND	TARGET	STRATEGY	Assessment
1-4	PrP^{SC}	Positive	Sequence/binding
5-6	$recPrP^{C}$	Negative	None
7-8	PrP^{SC}	Positive	Sequence/binding
9-10	$recPrP^{C}$	Negative	None
11-14	PrP^{SC}	Positive	Sequence

1.5 Conduct negative SELEX for bovine recombinant normal prion ($rPrP^{C}$) (Months 12-15)

Negative selection steps were performed against recPrP as outlined above. This negative selection is unique among SELEX strategies in that it utilizes an actual negative target (PrP^{C}) and not an immobilization substrate. Since our targets were free in solution, we had no immobilization substrate.

1.6 Assess enrichment of aptamer pool for target binding following round 6, 9, and 12 of SELEX (Months 9, 12, and 15)

Assessment of enrichment was performed primarily in two ways: analysis of sequences of selected clones for loss of heterogeneity and binding of immobilized targets using radiolabled aptamer pools.

We conducted binding experiments by probing nitrocellulose immobilized targets with radiolabled pool of aptamers from rounds 0, 4, and 8 (figure 11). The pools were labeled by amplifying an aliquot of each pool in the presence of α^{33}P-ATP with PCR. Labeling efficiency was estimated by PCR amplification using limiting amounts of unlabeled ATP, performing denaturing urea acrylamide electophoresis, staining with SYBR-gold to estimate total product, then exposure of radiographic film to estimate level of radioactivity (figure 12).

We also conducted binding experiments using a modified pull down technique in which the target (PrP^{SC}, recPrP, ssDNA binding protein, or BSA) was allowed to interact with the radiolabeled pool in solution, then the protein in solution was bound to a solid phase silica resin with free hydroxyl groups on the surface (Strataclean™ Resin from Stratagene). Samples were centrifuged and washed extensively. Total radioactive counts were measured as a representation of the amount of oligonucleotide bound to the target (figure 13).

Assessment of pool complexity (number of different oligonucleotides in a pool) was made by cloning selected pools into a TOPO TA cloning plasmid (Invitrogen). Briefly, this method uses a covalently bound topoisomerase to couple the overhanging 3' thymidine ends of a linearized vector to the overhanging 5' adenine residues that occur during PCR amplification. Screening was accomplished using a *LacZ* interruption strategy, white colonies picked for further analysis and sequencing. The sequences were aligned with an algorithm (Vector NTI™ from Invitrogen). The alignments were analyzed for development of sequence conservation (figure 14).

Objective 2 Sequence selected aptamers species and down-select for aptamer beacon engineering (Months 15-24)

2.1 Clone reduced aptamer pools from Objective 1.5 (Months 15-18)
2.2 Sequence aptamer clones (Months 18-21)
2.3 Assess sequence homologies from clones for family relationships (Month 21)
2.4 Determination of sensitivity and specificity of aptamers representative of aptamer families from 2.3 for CWD prion (PrP^{Sc}) (Months 21-24)
2.5 Ranking of sensitivity and specificity of aptamer families for CWD prion (PrP^{Sc}) (Month 24)

Objective 2 is dependent upon successful realization of objective 1. We have applied for a no cost extension of the research to continue our efforts toward selection of a specific aptamer for PrP^{SC}. To overcome the difficulties encountered using CWD PrP^{SC} isolated from infected tissue as our target for selection, we propose to use a novel antibody displaying selected motifs of PrP^{SC} that have been shown to be involved in prion protein mis-folding as our target for aptamer selection. Two such motif grafted antibodies for PrP sequences 89-112 and 136-158 were developed by Williams laboratory at the Scripps Research Institute [6]. The grafted motifs of these antibodies specifically recognize mis-folded PrP^{SC}, and do not recognize the native form of the prion protein. The Williams lab have graciously consented to collaborate with us using their PrP^{SC} specific motif grafted antibodies as targets for aptamer selection, and they have provided us with these antibodies through an MTA. Experiments are underway to select aptamers to the PrP^{SC} specific motifs displayed on these antibodies. A capillary electrophoresis aptamer selection protocol is being developed for the selection process.

Key Research Accomplishments

- Protocols for SELEX using cellulose immobilized peptides developed
- Four rounds SELEX accomplished against immobilized peptide YYR and reduced aptamer pool cloned in preparation for sequencing and assessment of aptamer pool reduction
- Crude elk CWD prion protein target prepared and assessed for use in SELEX
- Novel electrodialytic SELEX protocol developed for use with crude elk CWD prion protein
- Materials transfer agreement with USDA executed to obtain recombinant elk normal prion protein genetic construct

- Preparation of isolated recombinant elk normal prion protein
- Fifteen rounds SELEX utilizing recPrP and cervid PrP^{SC}
- Developed protocol for electrodialysis
- Developed protocol for binding assays using protein targets immobilized on nitrocellulose
- Developed protocol for pull down assay using protein targets free in solution
- Materials transfer agreement with Williams laboratory at the Scripps Research Institute for motif grafted antibodies to use as targets for selection

Reportable Outcomes

Presentations:
Blair JB, Clinkenbeard KD Selection of an Aptamer for Diagnosis of Chronic Wasting Disease. *National Prion Research Program Meeting* 2005, Washington, DC.

Clinkenbeard KD, Jean Clarke J, Malayer JR, Hancock LF, Moon JH, Guo N, Timothy A. Snider TA, Dye R, Wang S. Aptamers for Detection of Biowarfare Agents. *Army Research Office Workshop for on the Chip Detection of Biological and Chemical Molecules.* 2004, Raleigh, NC.

Jeff Blair, Selection of an Aptamer to the Misfolded Prion Protein of Chronic Wasting Disease Oklahoma State University Graduate College Research Symposium (03-05-2004)

Jeff Blair, Development of a Novel Electrodialysis Method for SELEX Oklahoma State University Graduate College Research Symposium (02-24-2005)

Jeff Blair, Kenneth Clinkenbeard, Aptamers as Diagnostic Tools for Transmissible Spongiform Encephalopathies, TSE in the Americas Conference, Ames, IA (10-07-2005)

Jeff Blair; Will Sims; Katherine I. O'Rourke[§]; Kenneth D. Clinkenbeard. Discovery Research to Select an Aptamer to the Mis-folded Prion Protein of Chronic Wasting Disease. Oklahoma State University CVM Phi Zeta Research Day (03-18-2004)

Blair JL, Clinkenbeard K, and O'Rourke K. Selection of an aptamer to the misfolded prion protein of chronic wasting disease. Oklahoma State University Food and Agricultural Products Research and Technology Center Symposium (04-19-2004)

Graduate Student(s) Supported: Jeffrey Blair, DVM enrolled in Veterinary Biomedical Sciences PhD program
Funding Applied for Based on this Work Supported by this Award: Small Business Technology Transfer (STTR) Program, Proposal Number: A045-027-0237; Topic Number: A04-T027; Ruminant B-Lymphocyte Yellow Fluorescent Protein Aggregation Bioassay for Elk Chronic Wasting Disease

Conclusions

The difficulty of the work and a four-month work stoppage for work with prion proteins have delayed the progress of the project. The long delay was the caused by a lack of

familiarity of our IBC with standard protocols for working with prions. The groundwork has been laid by developmental work for PCR optimization, production and purification of SELEX targets, immobilization strategies, aptamer pool cloning, and radiolabeling experiments such that we expect to move more quickly through the project objectives during Year 3. Once an aptamer is selected, it will be engineered as a signaling moiety for the rapid antemortem diagnosis of CWD. In addition, the strategies used in selection of this aptamer can be rapidly adapted to selection of aptamers for other TSE targets, such as BSE and CJD such that, once optimized, our strategy may result in an important step forward in the diagnosis of all of these important diseases.

We have also established important collaborative relationships with other prion disease research laboratories around the United States, including Dr. Katherine O'Rourke's laboratory at Washington State University, Dr. Bruce Chesebro's group at the National Institutes of Health Rocky Mountain Laboratory, Dr. Jason Bartz's laboratory at Creighton University, and Dr. Mike Miller at Colorado State University. Dr. Blair has visited each of these individuals with the purpose of establishing relationships and soliciting input on this project. We have also in place a materials transfer agreement with Dr. Williams laboratory at the Scripps Research Institute to utilized motif grafted antibodies as a target for aptamer selection. Our laboratory served as host from visits to OSU by Dr. O'Rouke (October 2004) and Dr. Bartz (February 2005). These colleagues have already proved invaluable for advice on our current project, and it is likely that these relationships will be very fruitful for future collaborative investigations between our laboratories.

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5. Paramithiotis, E. et al. A prion protein epitope selective for the pathologically misfolded conformation. Nat Med 9, 893-9 (2003).
6. Moroncini, G. et al. Motif-grafted antibodies containing the replicative interface of cellular PrP are specific for PrPSc. Proc Natl Acad Sci U S A 101, 10404-9 (2004).

Appendices

Figures

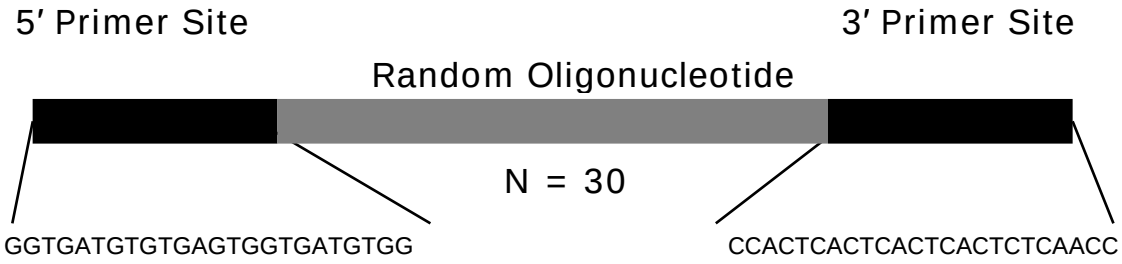


Figure 1 Depiction of aptamer construct. Random central 30mer depicted in grey.

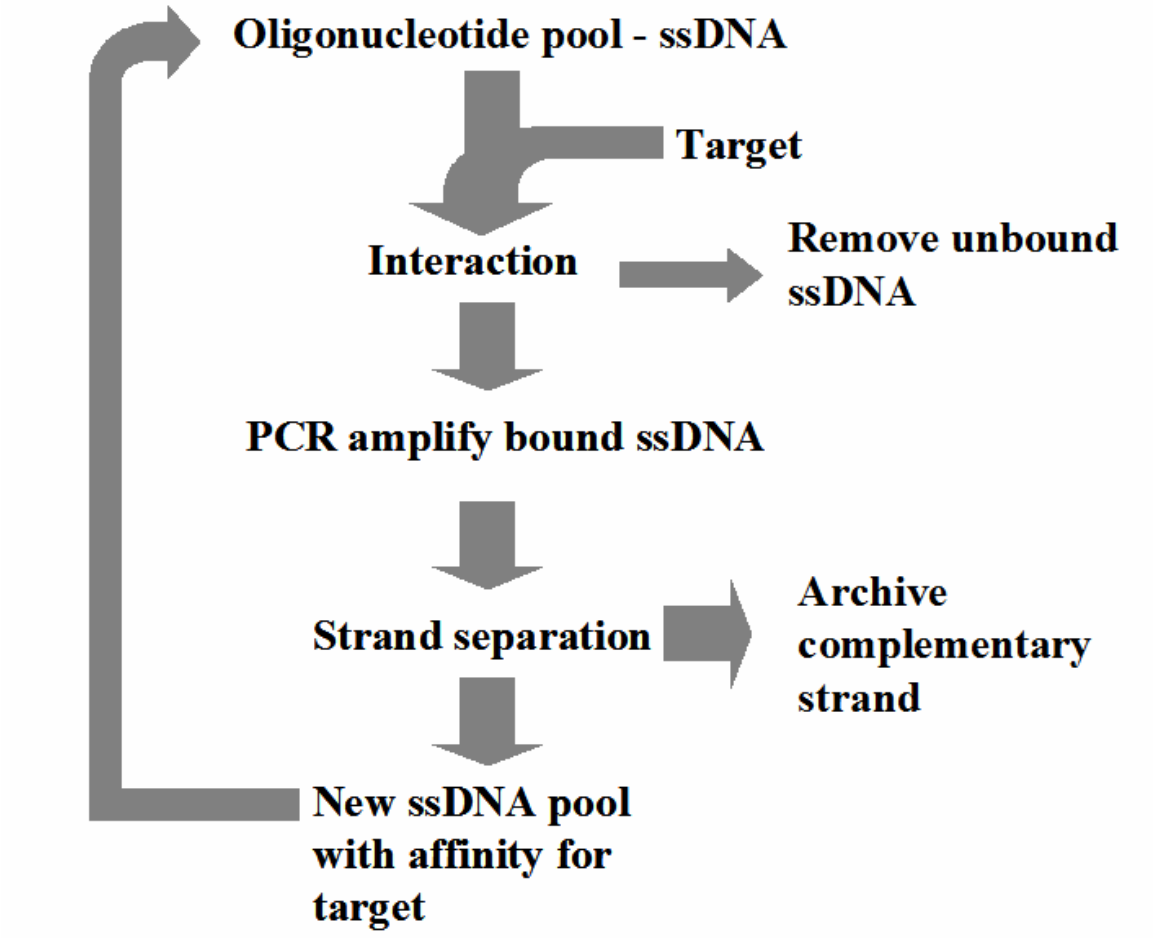


Figure 2 Illustration of SELEX

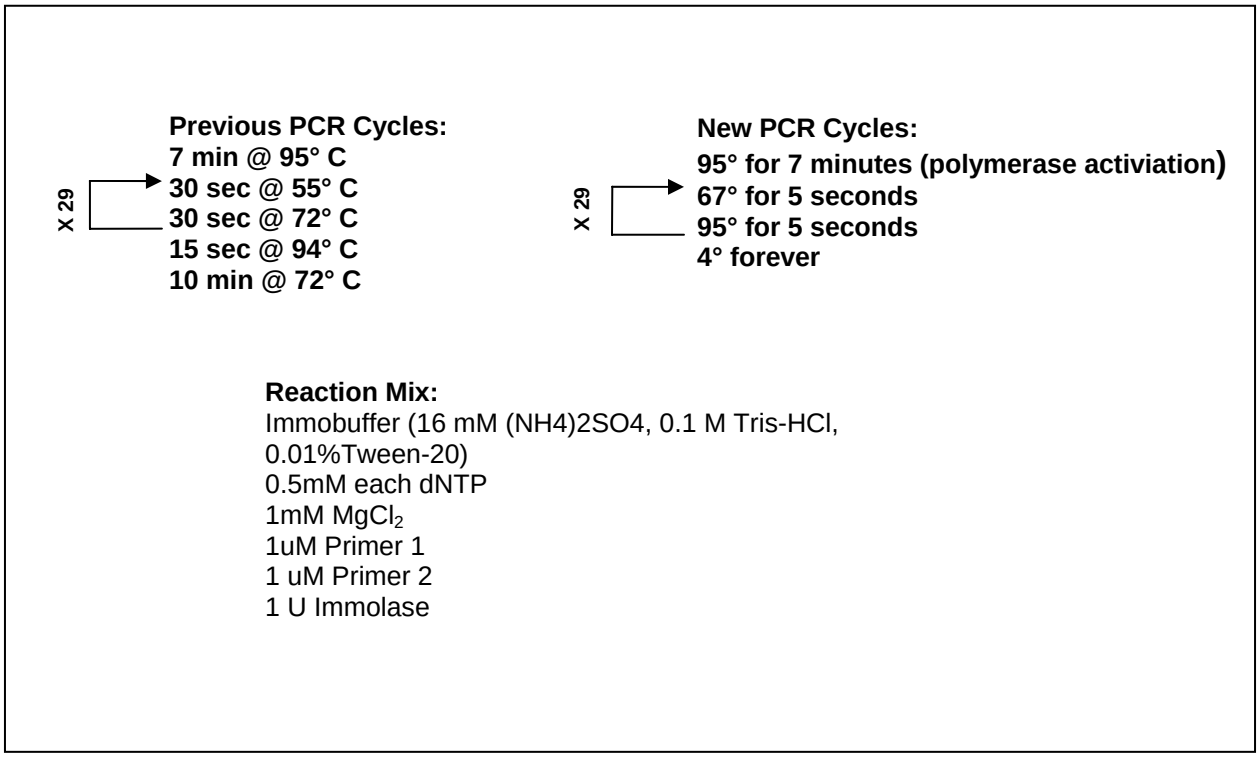


Figure 3 Comparison of optimized PCR reaction conditions

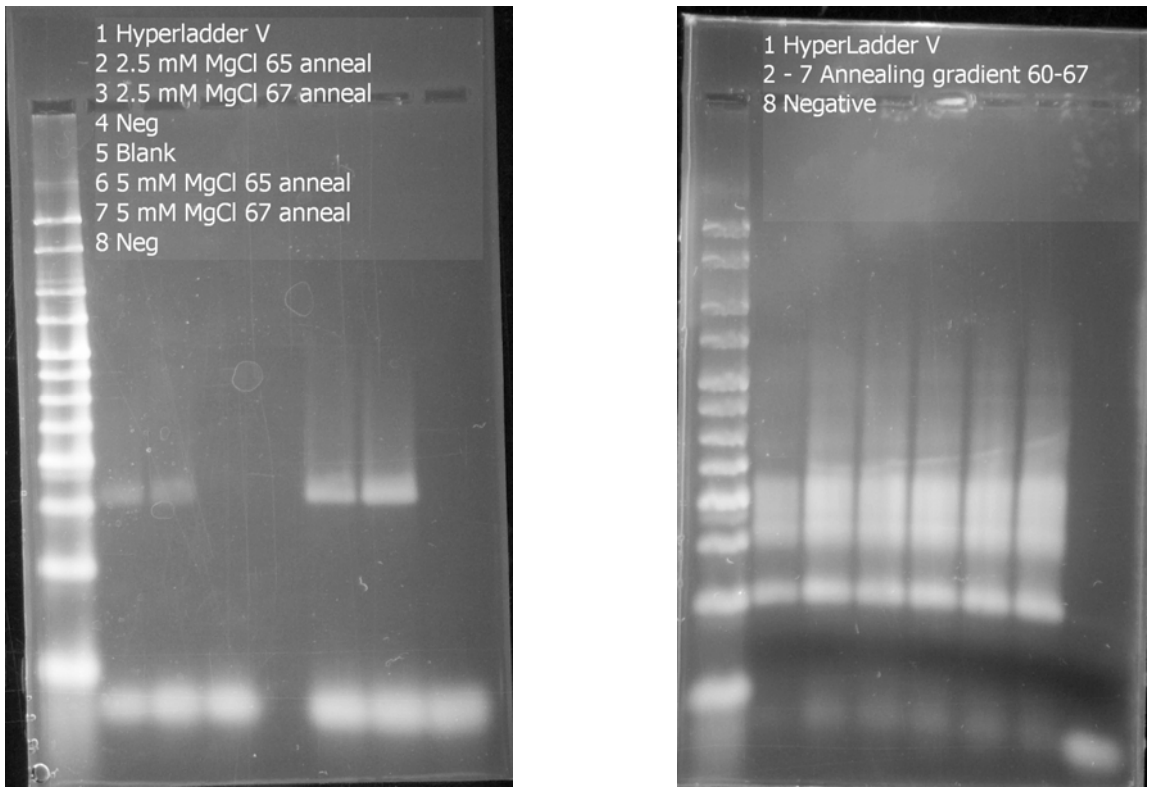


Figure 4 Comparison of products obtained by 2 step and 3 step PCR

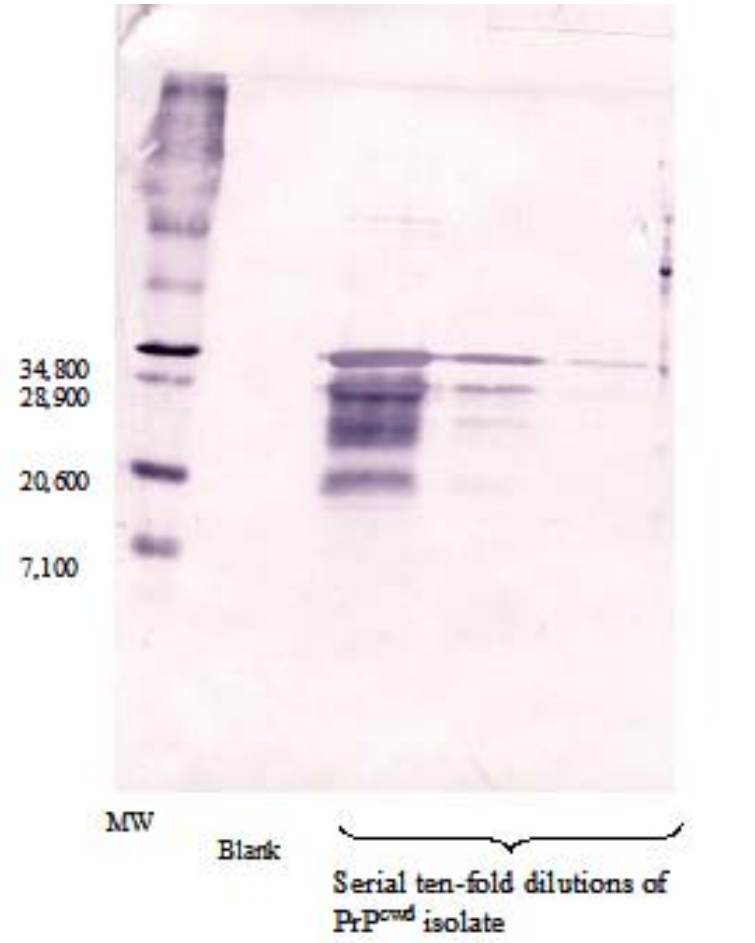


Figure 5 Western Blot of serial dilutions of PTA precipitation preparation probed with Mab 6H4

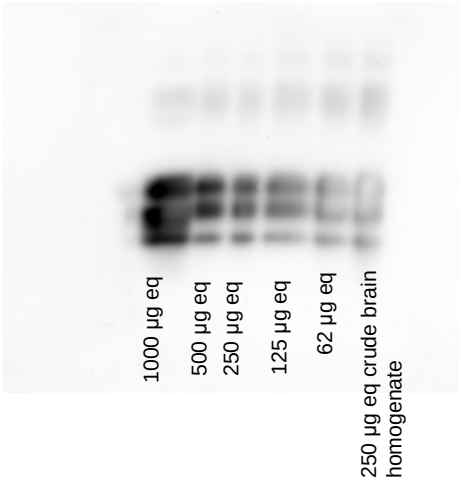


Figure 6 Western blot of Beeke's ultracentrifuge preparation probed with Mab 6H4

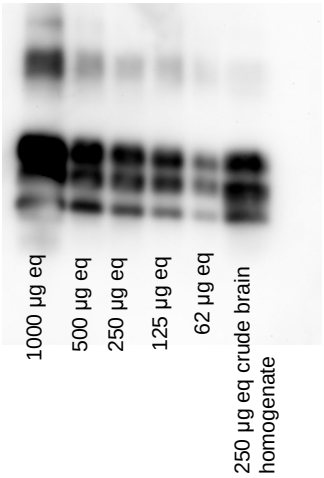


Figure 7 Western blot of Bolton's ultracentrifuge preparation probed with Mab 6H4

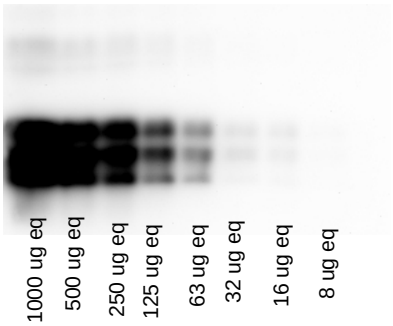


Figure 8 Western blot of crude brain homogenate serial dilution probed with Mab 6H4

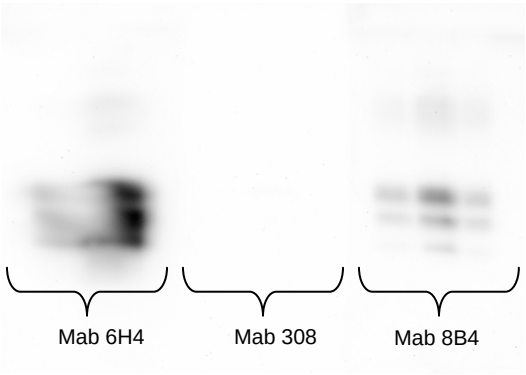


Figure 9 Serial dilutions of crude brain homogenate probed with different anti-PrP monoclonal antibodies

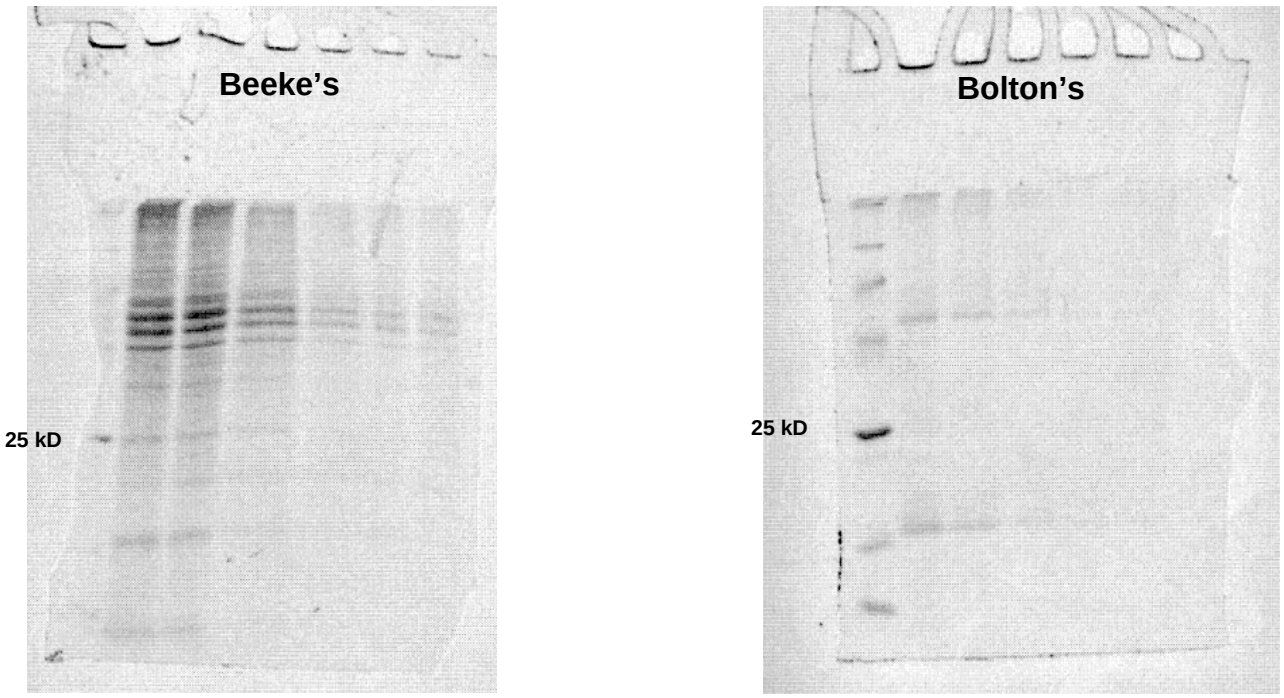
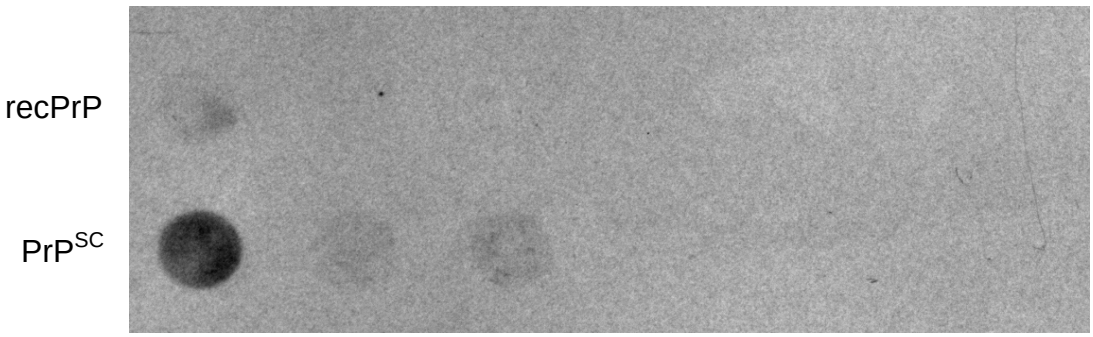
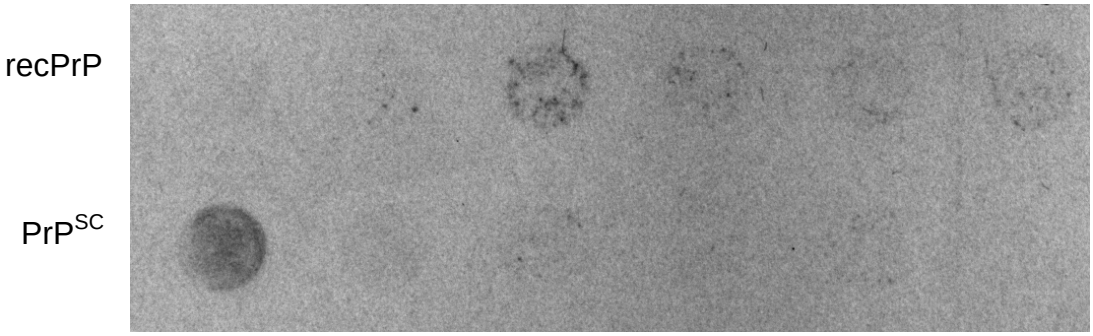


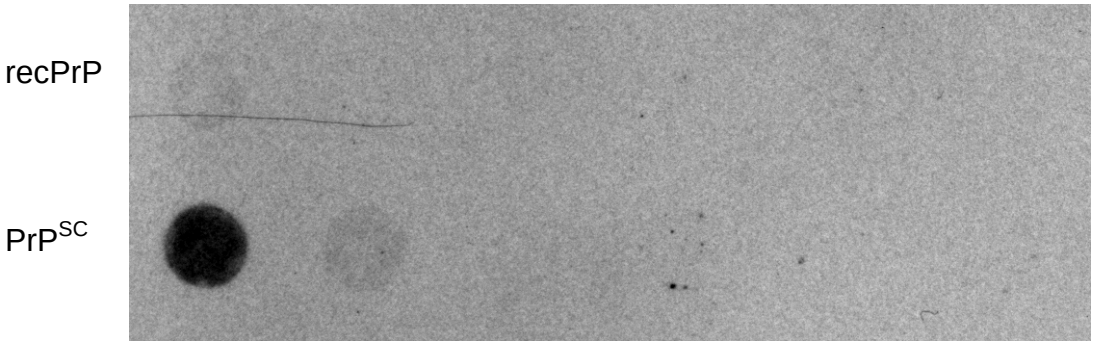
Figure 10 Serial dilutions of Beeke's (left) and Bolton's (right) ultracentrifugation method of preparing PrP^{SC} stained with SYPRO Ruby demonstrating relative amounts of protein in each preparation



A. Round 0



B. Round 4



C. Round 8

Figure 11. Dot blots of serial dilutions of recPrP and PrP^{SC} probed with radiolabeled aptamer pools. Serial dilutions are 10 μg PrP^{SC} (330 pmole), 2 μg (66 pmole), 0.4 μg **(**13.2 pmole), 0.08 μg (2.6 pmole), 0.016 μg (0.53 pmole), 0.004 μg (0.01 pmole). Equimolar amounts of recPrP were loaded in appropriate wells.

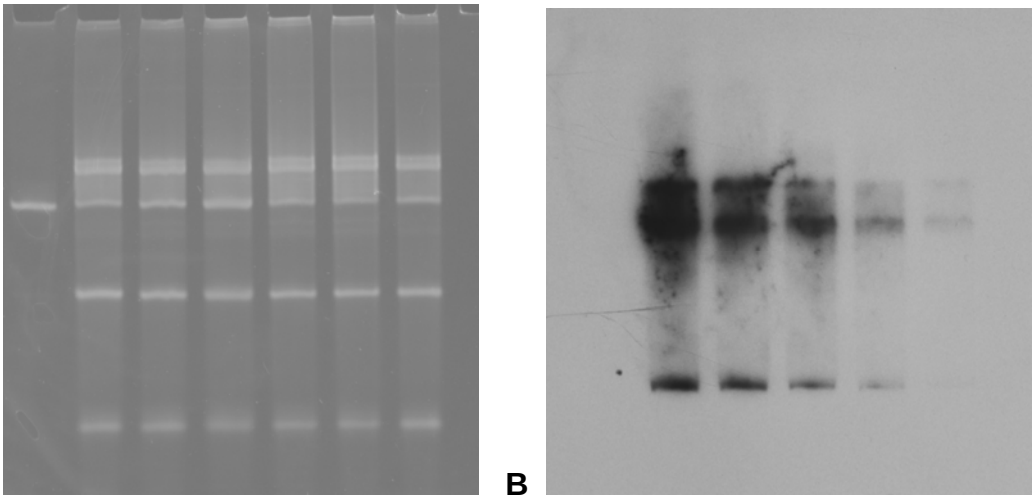


Figure 12 Optimization of pool labeling with α^{33}P-ATP. **A. Denaturing urea PAGE:** Lane 1 ssDNA marker (78 nt). Lane 2 standard reaction as control (no ^{33}P-ATP, 500 μM cold ATP). Lanes 3-8 varied concentration of total ATP (25μM, 50μM, 100μM, 250 μM, 500 μM). ^{33}P-ATP in all reactions was approximately 2 μCi (3.3 pM). Concentration of GTP, CTP, and TTP held at standard reaction concentration of 500 μM. **B. Radiographic film exposed to gel for 10 min:** no development in control lane, most efficient labeling at 25 μM

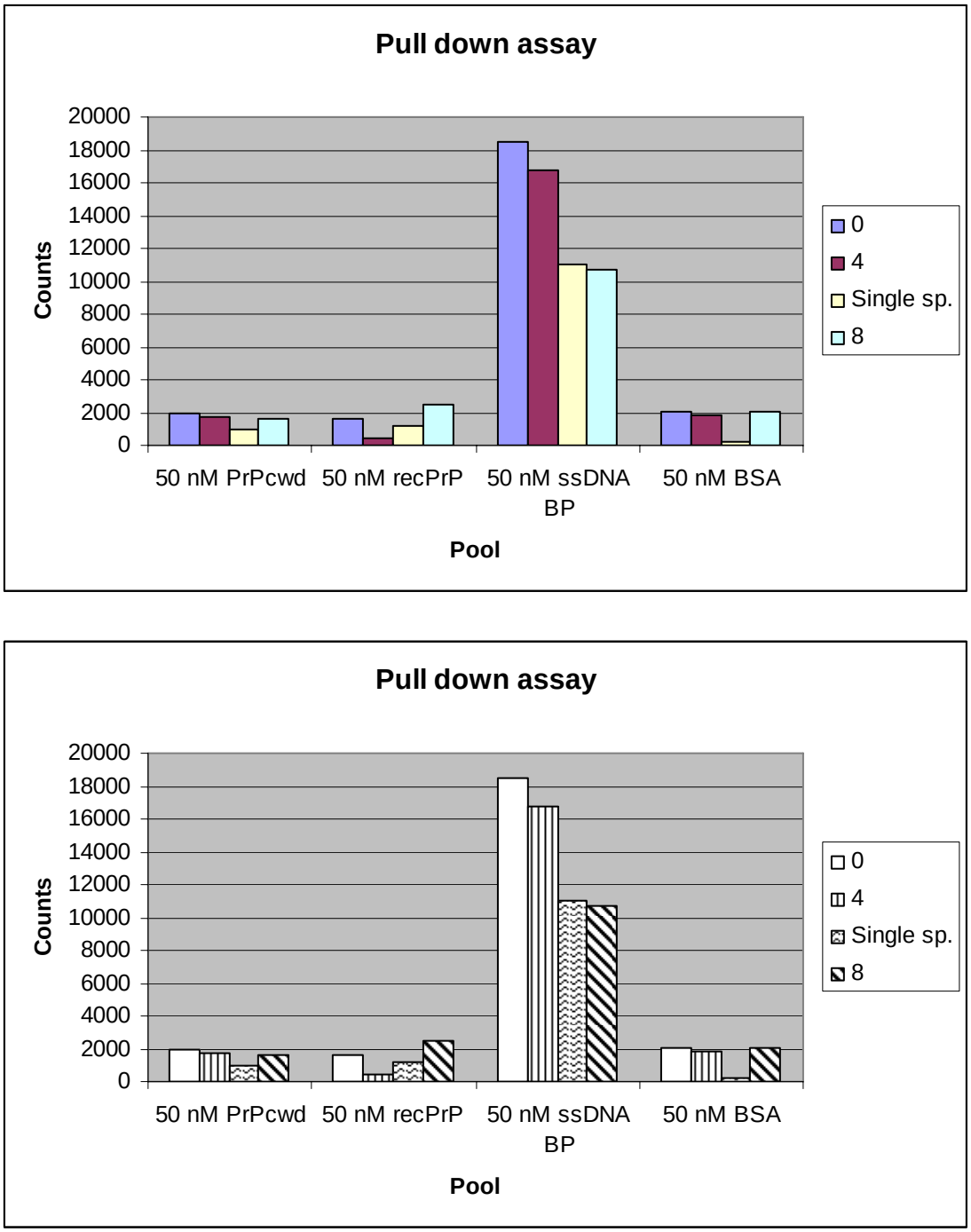
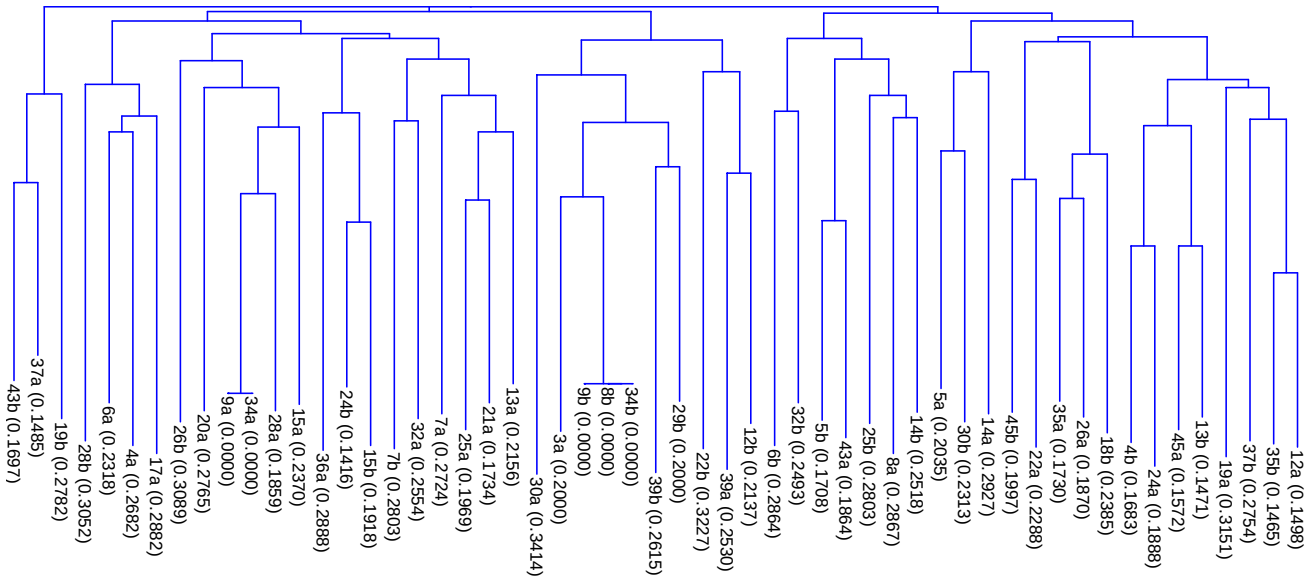
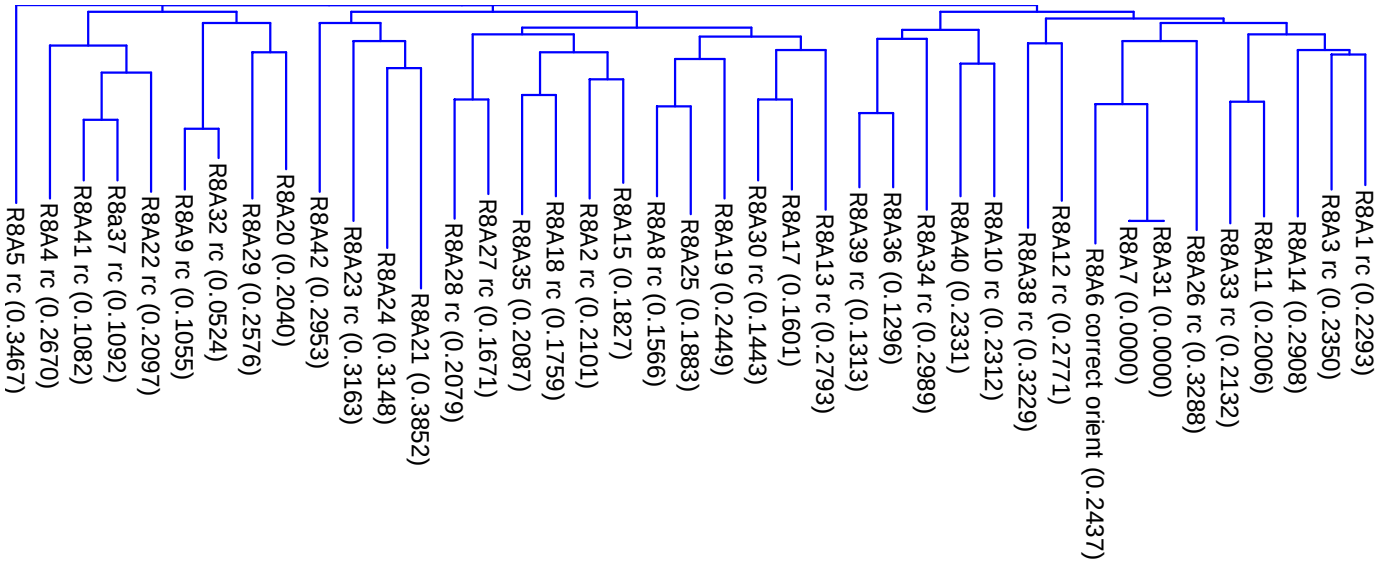


Figure 13. Comparison of counts of radiolabeled pools pulled down bound to protein targets. Single species is an amplified single clone from round 8 that occurred in duplicate. ssDNA BP is *E. coli* single stranded DNA binding protein.



A. Round 4 sequence alignment



B. Round 8 sequence alignment

C. Round 15 sequence alignment

Figure 14. Alignments of pools at round 4, 8, and 15. Positive selection would be indicated by two or three major families with obvious conservation of sequences. The heterogeneity represented by these alignments indicate that no conservation of binding sequences has occurred.