Appendix I

DNA Pulldown: Capture Purification of DNA Fragments with Hairpin Polyamide-Biotin Conjugates

Initial pulldown experiments using fluorescently labeled oligonucleotides were done in collaboration with postdoctoral scholar Alex Heckel.

Abstract

The purification and isolation of fragments of genomic DNA would be of great use to the biochemical field. We report herein efforts towards the use of DNA-binding polyamide-biotin conjugates to isolate targeted fragments of DNA from complex mixtures using streptavidin-coated magnetic beads. Experiments done in the presence of 2 kB of DNA indicate that the polyamide specificity remains an important hurdle to moving forward.

Introduction.

Chromatin Immunoprecipitation (ChIP) has emerged as a powerful technique for identifying protein-protein and protein-DNA interactions.[1-5] In this protocol, biological DNA-protein macrostructures are crosslinked inside cells with formaldehyde. The cells are then lysed and the DNA sheared into approximately 500 base pair fragments. An antibody targeted towards a protein that is known to bind to the sequence of interest is then used to purify that fragment (and all proteins crosslinked to it). The covalent crosslinks are then reversed, and the individual components of the complex purified by gel electrophoresis and analyzed by mass spectrometry. Using this technique, all elements of a promoter of a gene of interest can be identified.

CHiP does, however, suffer from several disadvantages. First, *a priori* knowledge of at least one of the proteins bound to the sequence of interest is a prerequisite for antibody generation. Second, antibody generation is itself a tedious process that does not always yield useful binders.

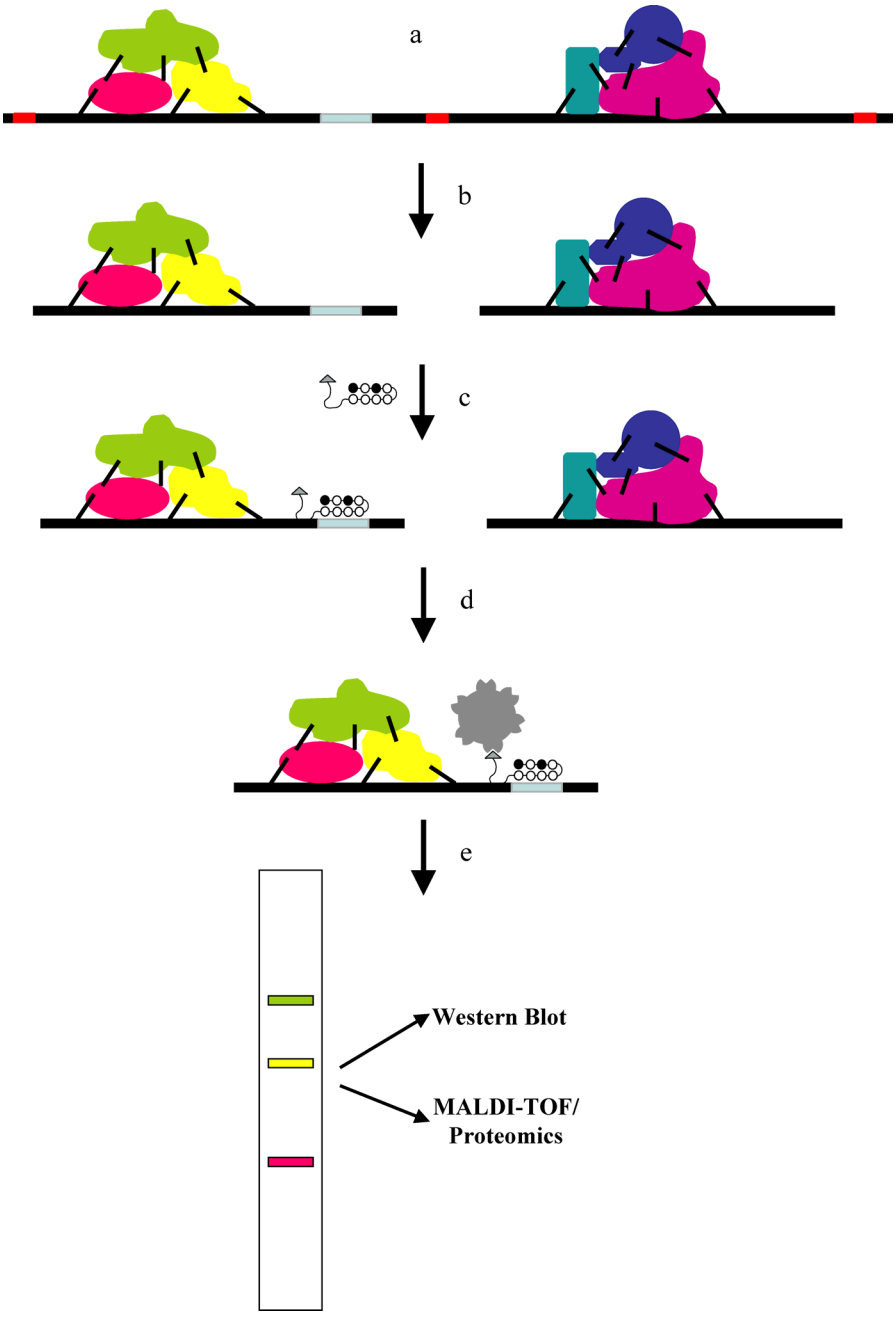
Over the past ten years, researchers in the Dervan lab have developed programmable small molecules capable of recognizing predetermined sequences of DNA with high affinity and specificity.[6-8] Furthermore, these polyamides, which bind in the minor groove of the DNA duplex, have been shown to bind DNA in the presence of nearby proteins, proteins bound to the same site but in the major groove, and even to DNA packaged on chromatin.[6, 9] Because of their ability to bind DNA (and even protein-DNA complexes) with affinities and specificities rivaling natural DNA-binding proteins, we explore the use polyamides as molecular tools for the isolation of specific DNA fragments from complex mixtures.[10]

Because of recent genome sequencing efforts, the base pair content of almost every human gene and promoter is known.[11] The identities of proteins bound to a given promoter at any given time are not as well understood. Because of precise sequence information, polyamides can be designed to target a promoter of interest. DNA-protein complexes can be crosslinked inside cells using formaldehyde, and the DNA sheared into 500 base pair fragments. By conjugating a chemical handle to a polyamide that is capable of binding the sequence of interest with high affinity and specificity, the sequence of interest, and all of its associated proteins, can be purified, or pulled down, from the mixture of oligonucleotide fragments.

The use of DNA-binding polyamides for this type of analysis has several advantages over CHiP. First, polyamides can be easily designed and synthesized to target almost any sequence of DNA. Second, polyamides become useful for studying promoters for which there are no known protein binders. We report herein progress towards the use of polyamides as "pulldown" reagents for DNA capture.

DNA Pulldown with Biotin-Polyamide Conjugates.

Many different pulldown architectures can be envisioned. For example, polyamides could be conjugated to a solid support, such as polystyrene or polyacrylamide beads. Alternatively, polyamides may be functionalized with a chemical handle that can later be recognized by a functionalized solid support. This method has the advantage that the polyamides can equilibrate with the DNA in solution, rather than on solid support, which may impede the equilibration process. We chose to use polyamide-biotin



Scheme I.1. Schematic procedure for isolating a locus of interest (left side) from genomic DNA. Genomic DNA is crosslinked to associated proteins using formaldehyde. (a). DNA is then fragmented (at red squares) either by shearing or restriction digest. (b). A polyamide-biotin conjugate designed for the locus of interest (blue square = binding site) is then incubated with the DNA pieces. (c). The fragments are then incubated with streptavidin-coated beads. After washing, only the locus of interest remains bound via the polyamide-biotin:streptavidin interaction. (d). The fragment of interest is then eluted from the beads, the formaldehyde crosslinks reversed, and the proteins analyzed by gel electrophoresis and mass spectrometry.

conjugates. The DNA-polyamide complexes can then be isolated from DNA fragments that do not bind the polyamide by incubation with streptavidin-coated beads. The biotin-streptavidin interaction is one of the strongest non-covalent interactions known. For these studies, streptavidin-coated magnetic beads are used (commercially available from Dynal, www.dynal.no). Thus, after immobilization of the polyamide:DNA complex on the streptavidin-coated beads, the supernatant can be facilely removed by using a magnet to collect and trap the bead complexes. Scheme I.1 illustrates a hypothetical pulldown experiment with polyamide-biotin conjugates.

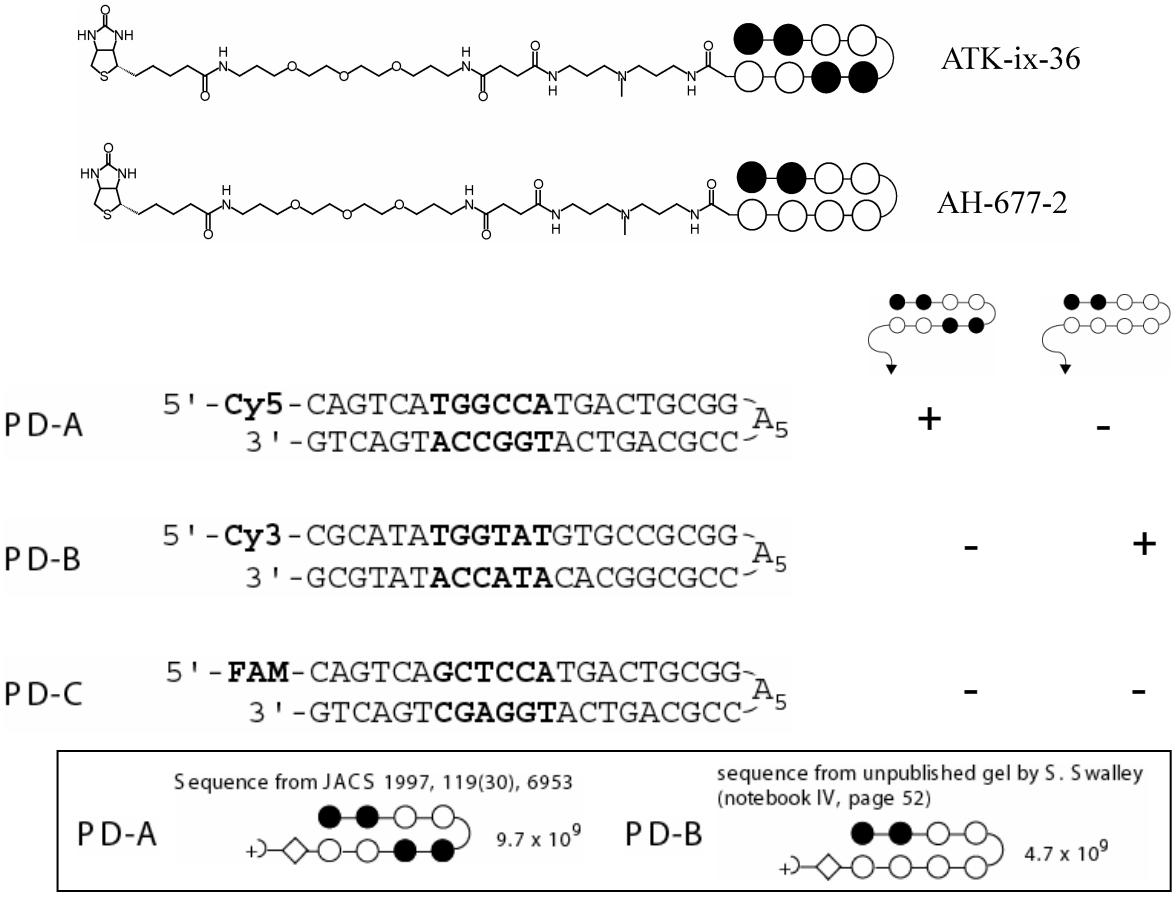


Figure I.1. Top: Compounds used in initial pulldown experiments. Black and white circles represent imidazole and pyrrole carboxamides, respectively. Bottom: Fluorescent duplexes used for pulldown studies. **PD-A** contains a match site for **ATK-ix-36**, **PD-B** contains a match site for **AH-677-2**, **PD-C** is a negative control. Boxed are measured binding affinities for the compounds (non-conjugated) used in this study. Plus represents match binding site, minus represents mismatch binding site.

The focus of initial experiments is to determine whether polyamide-biotin conjugates can isolate specific fragments of DNA from a mixture containing other fragments with formal mismatch binding sites. Polyamides **ATK-ix-36** and **AH-677-2** (synthesized by Alex Heckel) were synthesized by reacting polyamide amines with the commercially available biotinylation reagent TFP-PEO-Biotin (Pierce) (Figure I.1). Additionally, three duplex DNA oligonucleotides were synthesized containing match binding sites for **ATK-ix-36** (**PD-A**), **AH-677-2** (**PD-B**), and a negative control double mismatch site (**PD-C**) (Figure I.1). The three oligonucleotides were tagged with fluorophores with distinct excitation and emission spectra. This allows for the separation of each component's signal from a solution containing all three dyes, thereby allowing the amount of each oligo to be quantified.

Initial experiments to explore the specificity and yields of polyamide pulldown were then performed. Equilibrations and pulldown reactions are performed in 50 μL reaction volumes of TKMC/T20 (10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, 0.1% v/v Tween 20). An equimolar mixture of the three duplex oligonucleotides was incubated with a biotinylated polyamide (either **ATK-ix-36** or **AH-677-2**) for 15 minutes at room temperature. This equilibration time was shown to be sufficient to establish maximum binding selectivity.[12] After equilibration, streptavidin-coated magnetic beads were added, and the suspension shaken (600 rpm) for 15 minutes at room temperature. Again, this time was shown to be sufficient to maximally absorb all biotin complexes to the beads.[12] The pulldown capacity of the magnetic beads was experimentally determined to be 100–500 pmol biotin per μg bead.[12] After the incubation, the beads were concentrated and immobilized with a magnet, and the

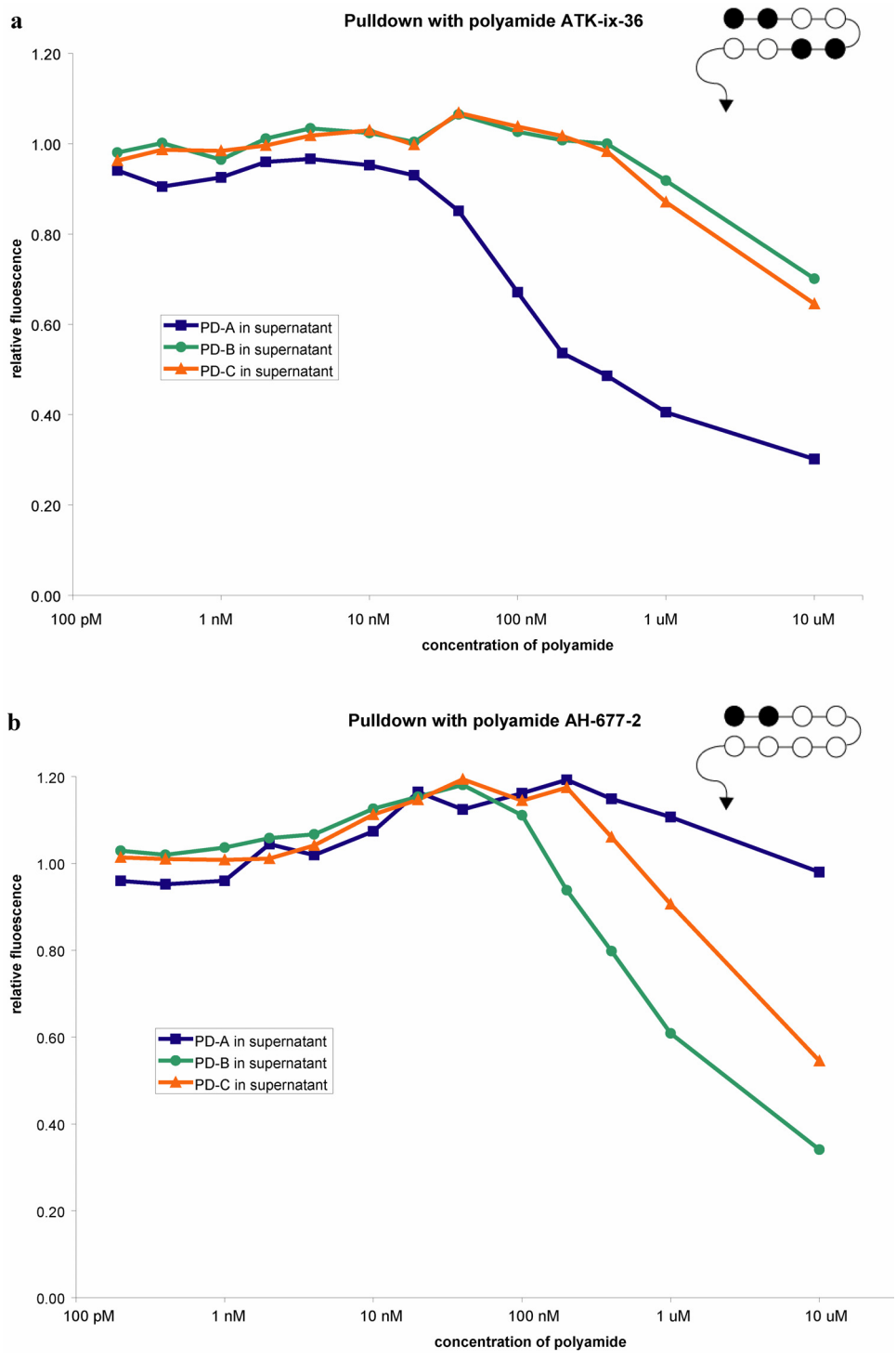


Figure I.2. a. Pulldown results with **ATK-ix-36**. **PD-A** is the match oligo, **PD-B** and **PD-C** are mismatch oligos. b. Pulldown results with **AH-677-2**. **PD-B** is the match oligo, **PD-A** and **PD-C** are mismatch oligos.

supernatant removed. The beads were then washed once with a fresh aliquot of buffer. Control vials in which no magnetic beads were added and vials in which no polyamide-biotin conjugate was added were run side-by-side with the pulldown reactions. 20 μL of each solution (control, supernatant, wash) was placed into wells on a 96-well plate. The plate was placed on a Typhoon phosphorimager and fluorescence was detected in each well. Using the ImageQuant software package, pulldown yields and efficiencies were calculated according to the following equation.

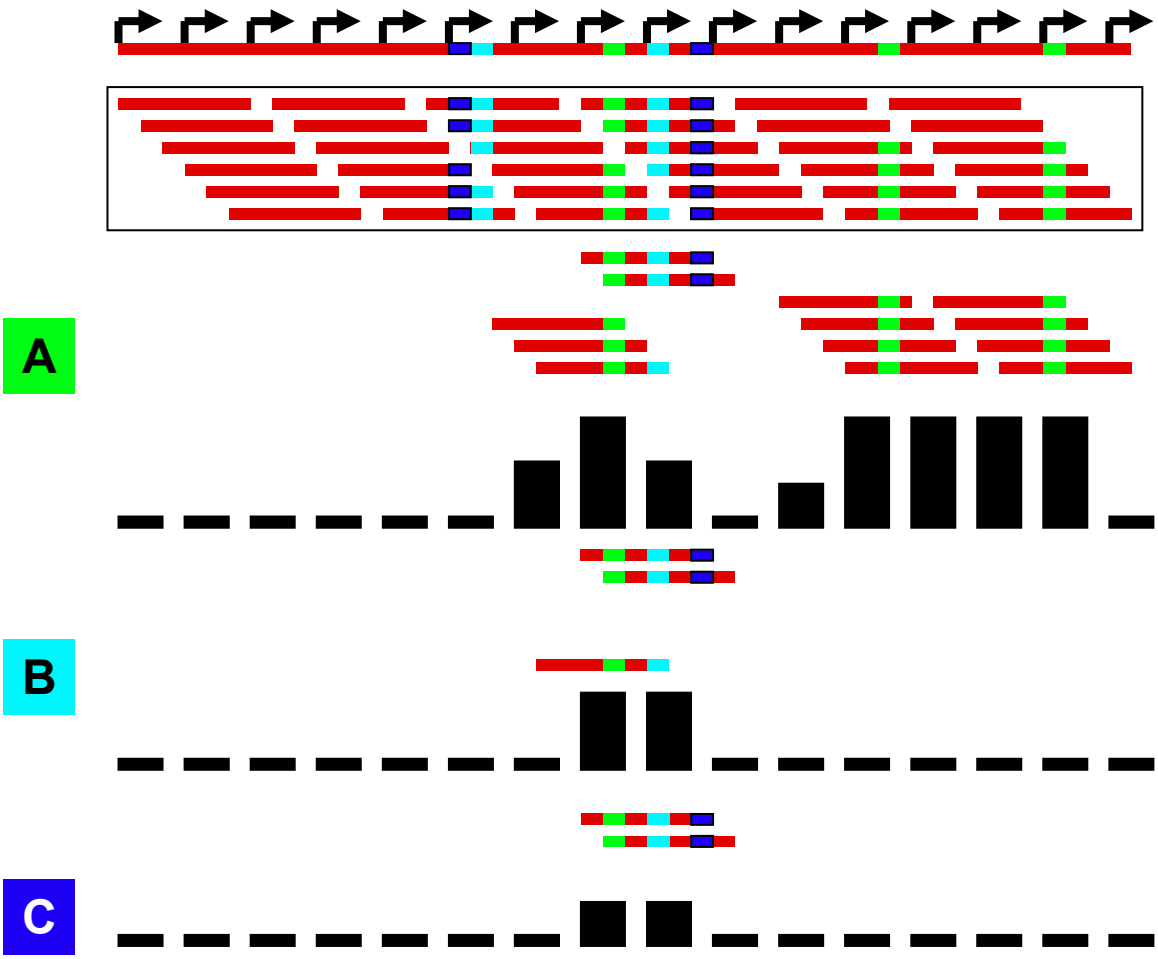
Pulldown yield = 1 / [((fluorescence in supernatant + wash)-2 x background)/(fluorescence in control-background)] x 100

Because polyamide binding and sequence specificity are dependent upon concentration, initial pulldown experiments were carried out over 5 orders of magnitude in concentration to determine the optimal concentration for both pulldown yield and selectivity for match oligo. Data for **ATK-ix-36** and **AH-677-2** are illustrated in figure I.2. As shown, at 300 nM, **ATK-ix-36** is able to capture 55% of **PD-A**, the duplex containing its match binding site, while leaving more than 95% of the two mismatch oligos (**PD-B** and **PD-C**) in solution. Even at 1 μM, **ATK-ix-36** is relatively specific, capturing 60% of **PD-A**, 4% of **PD-B**, and 3% of **PD-C**. At 300 nM, **AH-677-2** is less efficient, capturing only 20% of its match oligo **PD-B**. **AH-677-2** is also less specific, pulling down 7% of **PD-C** at 300 nM. At 1 μM, this polyamide captures 38% of match oligo **PD-B**, 25% of **PD-C**, and 7% of **PD-A**. While yields at 100 nM are not maximal, higher concentrations of polyamide-biotin conjugates led to significant non-specific pulldown. Thus, for future experiments, concentrations of 100 nM were used.

Release of Captured DNA.

Because our eventual goal is to use this technology to purify interesting DNA fragments, it is necessary to be able to recover the DNA of interest from the streptavidin-coated beads. Furthermore, our interest in this project is to purify large pieces of DNA from fragmented genomes. On the genomic scale, a typical 8-ring hairpin polyamide binding site can be found approximately one million times. Thus, we anticipate that in order to purify sequences from genomic DNA, multiple pulldowns will have to be performed. That is, in a genome that has been fragmented into approximately 500 base pair pieces, an 8-ring hairpin polyamide-biotin conjugate may pull out dozens of fragments where match sites are present and accessible. In order to fully isolate a single fragment, a second (and possibly a third, etc.) pulldown with a different polyamide will be done. Now, because the second pool contains fewer fragments, the probability of multiple fragments having an additional common sequence is reduced, and the fragment of interest can be further purified (Scheme I.2).

Initial attempts to disrupt either the biotin-streptavidin interaction or the polyamide-DNA interaction to release the captured DNA were unsuccessful. These include treatment with 3M NaOAc, pH 5.2, 37 ºC, 10 h; 4M NaCl, 37 ºC, 14 h; 50% formamide$_{(aq)}$, 90 ºC, 10 min; Chloroquine (intercalator), rt, 2 h; 1M guanidinium chloride, 37 ºC, 1 h; or N-methylamino dipropylamine in water, 37 ºC, 14 h.[12] While heating to 90 ºC did release ~30% of the captured DNA, this low yield coupled with incomplete pulldown led us to explore other options. In a final attempt, a solution of excess biotin was able to release ~90% of the captured DNA from beads. However, this leaves the polyamide-biotin conjugate bound



Scheme I.2. Schematic representation of how multiple pulldowns with different polyamides can be used to purify single fragments. Genomic DNA is fragmented (Boxed). Polyamide A is used to pulldown all fragments with match sites (green box). The fragments of interest (pieces with 3 binding sites) are now enriched, but impurities (all fragments on the right) are still present. Subsequent pulldown with either polyamide B or C results in the isolation of only the fragments of interest.

to the DNA, and attempts to extract the polyamide from the DNA were unsuccessful.

Desthiobiotin and iminobiotin are two commercially available biotin analogs that have reduced affinity for streptavidin. Desthiobiotin binds streptavidin with 100,000-fold lower affinity than biotin. Iminobiotin is a switchable streptavidin binder, associating tightly with streptavidin at pH > 9.0, but dissociating under acidic conditions due to protonation of the imine. Analogs of **ATK-ix-36** containing desthiobiotin and

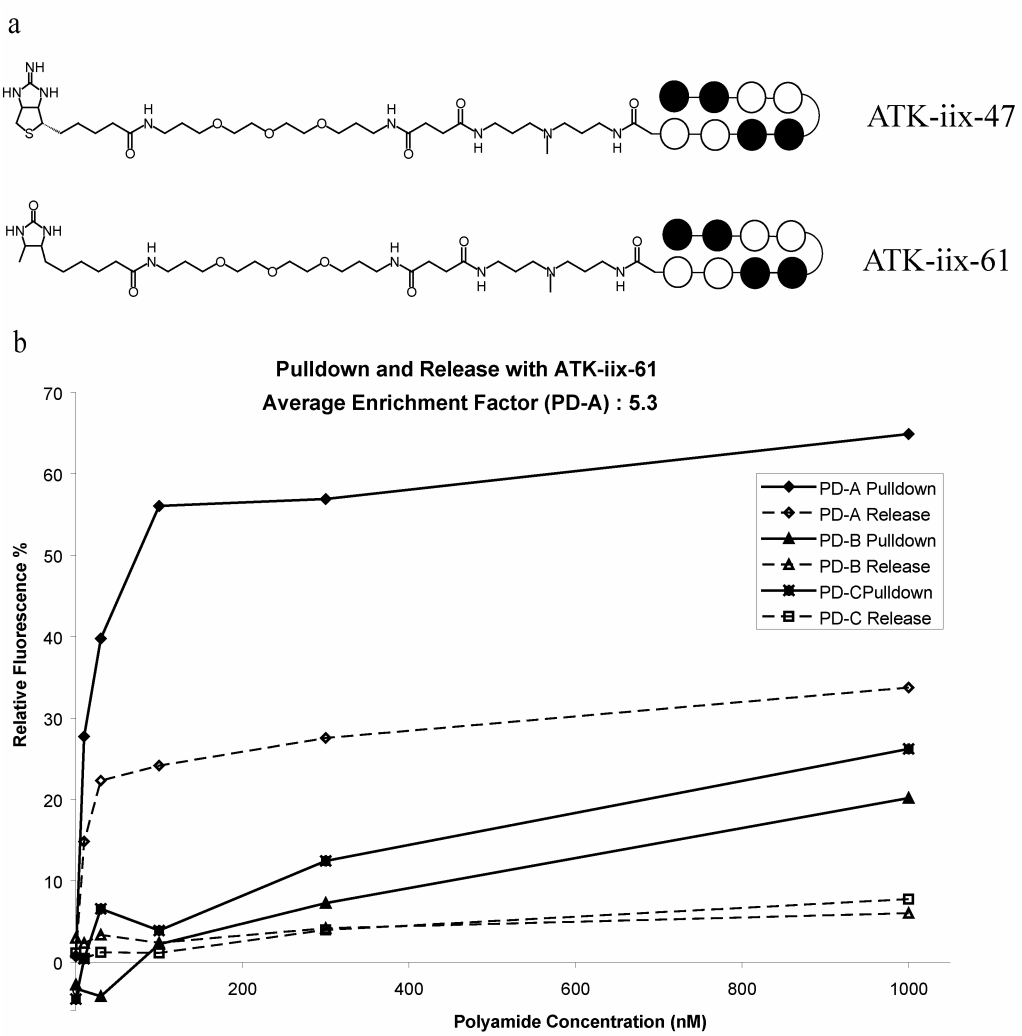


Figure I.3. a. Structures of polyamides conjugated to biotin analogs. **ATK-iix-47** was unable to capture significant amounts of DNA. b. Pulldown and release data for polyamide desthiobiotin conjugate **ATK-iix-61** (release done by incubating beads (with captured oligos) with 1 mM biotin (0.1 % Tween 20).). Enrichment factor calculated by dividing the amount **PD-A** by the amount of **PD-B** or **PD-C** at 100 nM.

iminobiotin were synthesized and tested for their ability to pulldown and release DNA (Figure I.3). Iminobiotin conjugate **ATK-iix-61** was able to pull down 35% of **PD-A** at pH 9.0. However, during the initial washing of the beads, 60% of the captured DNA was eluted from the beads. When the beads were treated with excess biotin for 1 hour, the remaining fraction of captured DNA was eluted. Desthiobiotin conjugate **ATK-iix-47** was able to capture more than 54% of the match oligo. Treatment with 1 mM biotin for 1 hour was able to displace approximately 50% of the captured oligo, resulting in a 25%

yield of **PD-A** after release. **PD-B** and **PD-C** were present in the release fraction in 4%, and 5% of their initial concentrations. Thus, **PD-A** was enriched by ~5-fold over each of the two mismatch oligos after one pulldown and release experiment.

Release with Cleavable Linkers.

Capture and release strategies with the biotin analogs described above still suffer from serious drawbacks. When the desthiobiotin-polyamide is used, upon release the conjugate is still associated with the DNA of interest. If it becomes necessary to perform

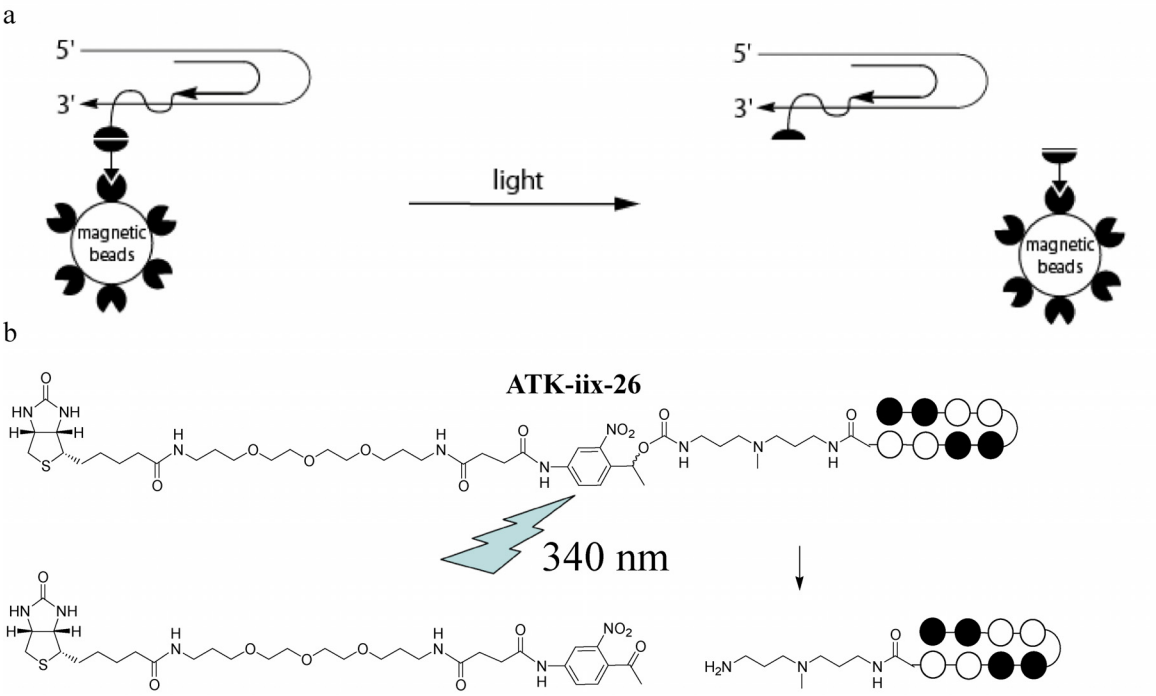


Figure I.4. a. Schematic representation of the incorporation of a photocleavable linker into a polyamide-biotin pulldown reagent. b. Chemical structures of the photocleavable polyamide-biotin conjugate **ATK-iix-26** synthesized for this project (top), and the putative structures after irradiation with 340 nm light.

multiple pulldowns to fully purify a fragment, this will lead to problems. If a second polyamide conjugate is added to the partially purified mixture isolated from a first pulldown experiment, the first pulldown polyamide is still present and no further purification can be achieved. We thus began exploring strategies to cleave the linker

between the DNA-binding portion and the streptavidin-binding portion of the capture agents. *Ortho*-nitro benzyl esters can be cleaved by irradiation with 340 nm light (Figure I.4). We set out to synthesize conjugate **ATK-iix-26**, which contains this photocleavable linker (Figure I.5). Amine **5** was synthesized according to published procedures.[13] This amine was then conjugated to PFB-PEO-Biotin (Pierce) to yield conjugate **6**. Reduction

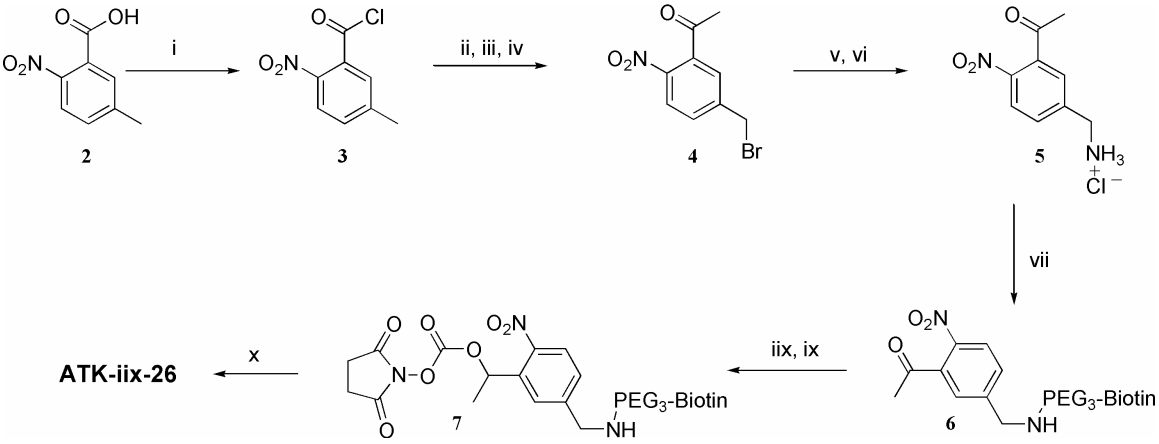


Figure I.5. Synthesis of photocleavable polyamide-biotin conjugate. i) $SOCl_2$; ii) $Mg[CH(COOEt)_2]_2$; iii) H^+/H_2O; iv) NBS, benzoyl peroxide; v) hexamethylene tetramine; iv) HCl, EtOH; vii) PEG_3-Biotin acid, DCC, HOBt; iix) $NaBH_4$; ix) Succinic anhydride, THF; x) ImImPyPy-γ-ImImPyPy-NH_2, DIEA, DMF.

of the ketone and subsequent NHS activation were accomplished according to published procedures.[13] Finally, the activated biotin linker **7** was conjugated to the tail amine of polyamide **ATK-ix-35** to yield **ATK-iix-26**.

Control experiments were done to determine photocleavage efficiency. 100 nM solutions of **ATK-iix-26** were irradiated with a handheld UV lamp at 365 nm for 10 minutes, 1 hour, 2 hours, and 4 hours. Cleavage was monitored by reversed phase HPLC (Figure I.6). As shown, complete cleavage was achieved after 2 hours of UV irradiation. It should be noted that optimal photocleavage is achieved at 340 nm. The literature reports complete cleavages in as little as 5 minutes when high-powered sources of 340

nm light are used.[13] Because initial control experiments were successful, a pulldown reaction was carried out using **ATK-iix-26.**

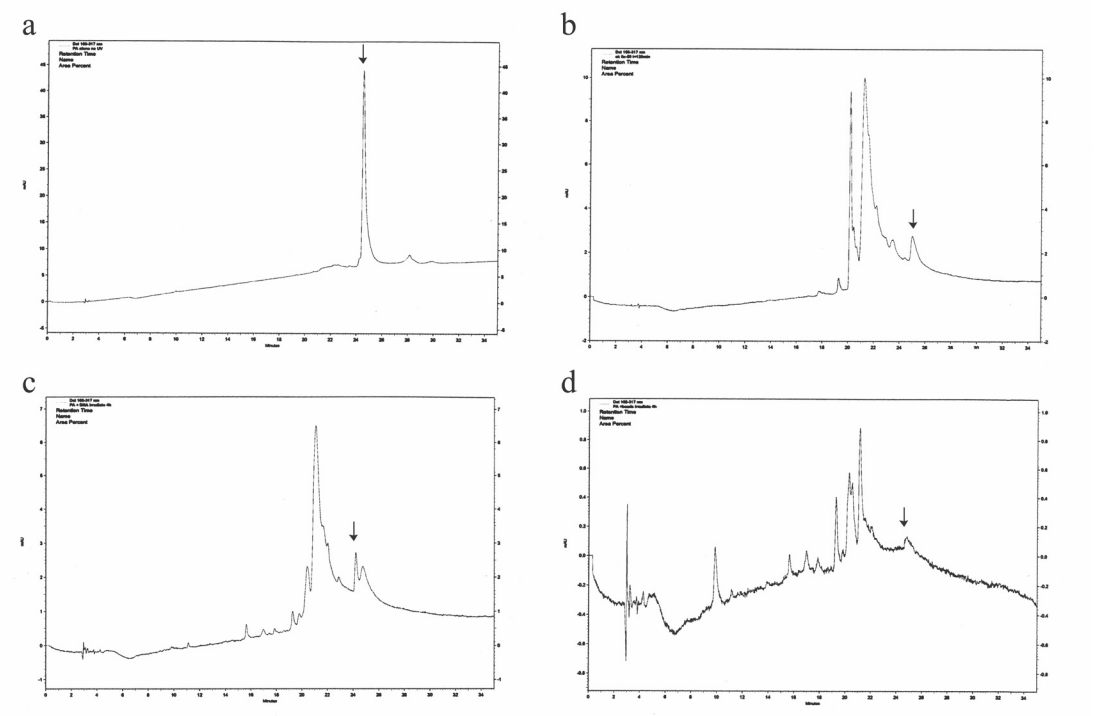


Figure I.6. Analytical HPLC traces monitoring the photocleavage of **ATK-iix-26**. a) Purified **ATK-iix-26**. In each trace, an arrow indicates the starting material. b) After 2 hours of irradiation with 365 nm light. Cleavage products are the two large peaks. c) 2 hour cleavage in the presence of 1 molar equivalent match DNA, linker is still efficiently cleaved. d) 2 hour cleavage in the presence of streptavidin-coated beads. Scale on this trace is 1/10 that of the other three traces, showing that yields for the photocleavage reaction on solid support are very low.

While 50% of the match DNA (**PD-A**) was captured by this conjugate, release by treating with 365 nm light for 4 hours did not yield any product. Controls were done to verify that the Cy5 dye on **PD-A** does not photobleach upon irradiation with 365 nm light. As an additional control, **ATK-iix-26** was absorbed onto streptavidin-coated beads, and irradiated with 365 nm light. As shown in Figure I.6, HPLC traces reveal very little polyamide in solution, and the polyamide suffers from degradation. Thus, photocleavage on solid support is not a feasible solution for release of captured DNA.

In a final attempt to solve the release problem, conjugate **ATK-iix-89**, which contains a chemically cleavable disulfide bridge, was synthesized (Figure I.7a). Pulldown experiments with this linker were performed as described above. Following

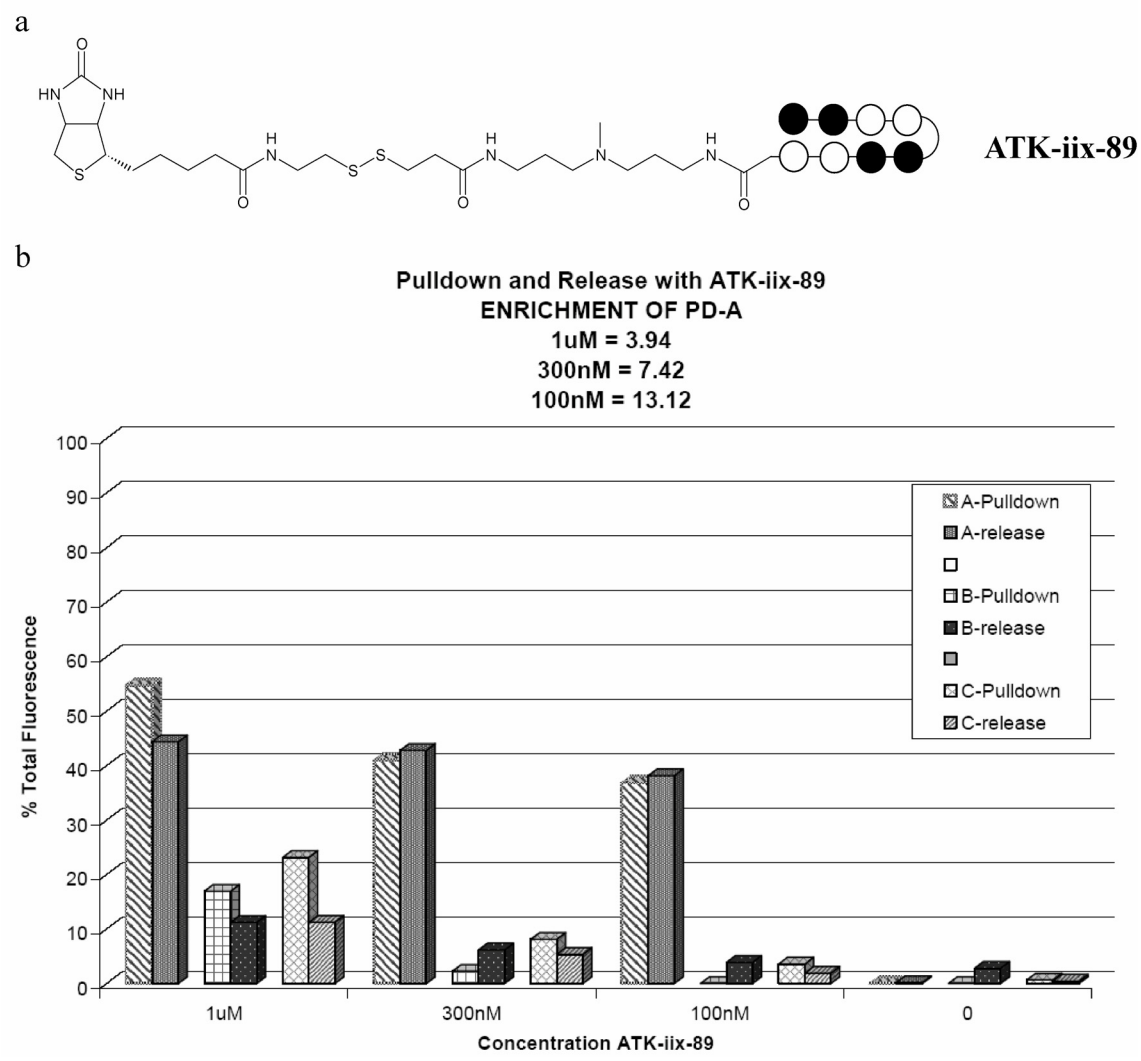


Figure I.7. a) Structure of chemically cleavable polyamide-biotin conjugate **ATK-iix-89**. b) Graphical representation of pulldown and release reaction with **ATK-iix-89**. Enrichment factors were calculated by dividing the amount of **PD-A** by the amount of **PD-B** or **PD-C**.

pulldown, release was achieved by first washing the magnetic beads with buffer, and then incubating in 50 mM dithiothreitol (DTT) for 30 minutes at 37 ºC to cleave the disulfide bond. Figure I.7b shows the results of these experiments. Conjugate **ATK-iix-89** is able

to capture almost 40% of its match DNA (**PD-A**) with less than 5% impurity at 100 nM. Furthermore, treatment with DTT is able to release 100% of the captured DNA. Thus, after a single pulldown, **PD-A** is able to be purified from **PD-B** and **PD-C** in 40% yield with a 13-fold enrichment factor.

Towards Large Fragment Pulldown

Because an efficient pulldown and release strategy had been elucidated, we were next interested in whether this strategy could be used to purify larger sequences of DNA. pUC19 is a 2686 base pair circular plasmid. A survey of restriction enzyme sites on pUC19 was made to find a restriction enzyme that would create a library of DNA fragments ranging from 100 base pairs to 1000 base pairs. MspA1 I (New England Biolabs) was identified as a restriction enzyme that fulfills the above requirements

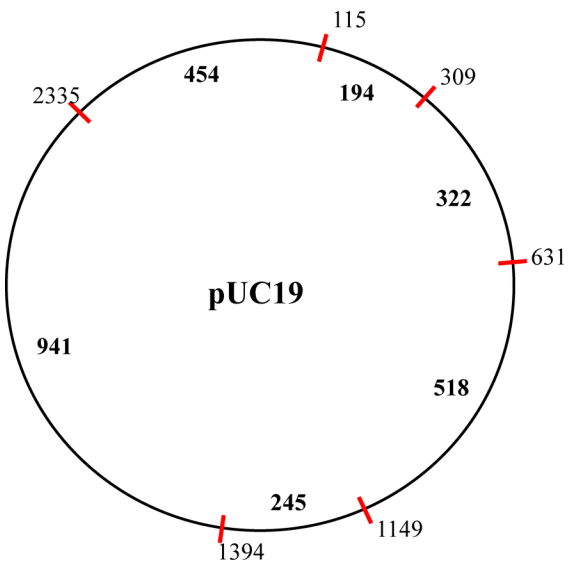


Figure I.8. Schematic representation of pUC19 digested with the restriction enzyme MspA1 I. Digestion at the indicated cut sites (red lines, with the base pair position of cleavage indicated outside the circle) yields 5 fragments ranging from 941 to 194 base pairs (fragment sizes indicated on the interior of the circle).

(Figure I.8). Cutting pUC19 with MspA1 I yields 6 fragments ranging from 194 to 941 base pairs. Additionally, the six lengths are all distinct from each other and can thus be individually visualized by non-denaturing gel electrophoresis. Pulldown experiments were then performed on a mixture of these six fragments using disulfide-bridged

polyamide-biotin conjugates. Briefly, the mixture of oligos was incubated with polyamide conjugate for 10 hours in TKMC/T20 buffer. Streptavidin-coated magnetic

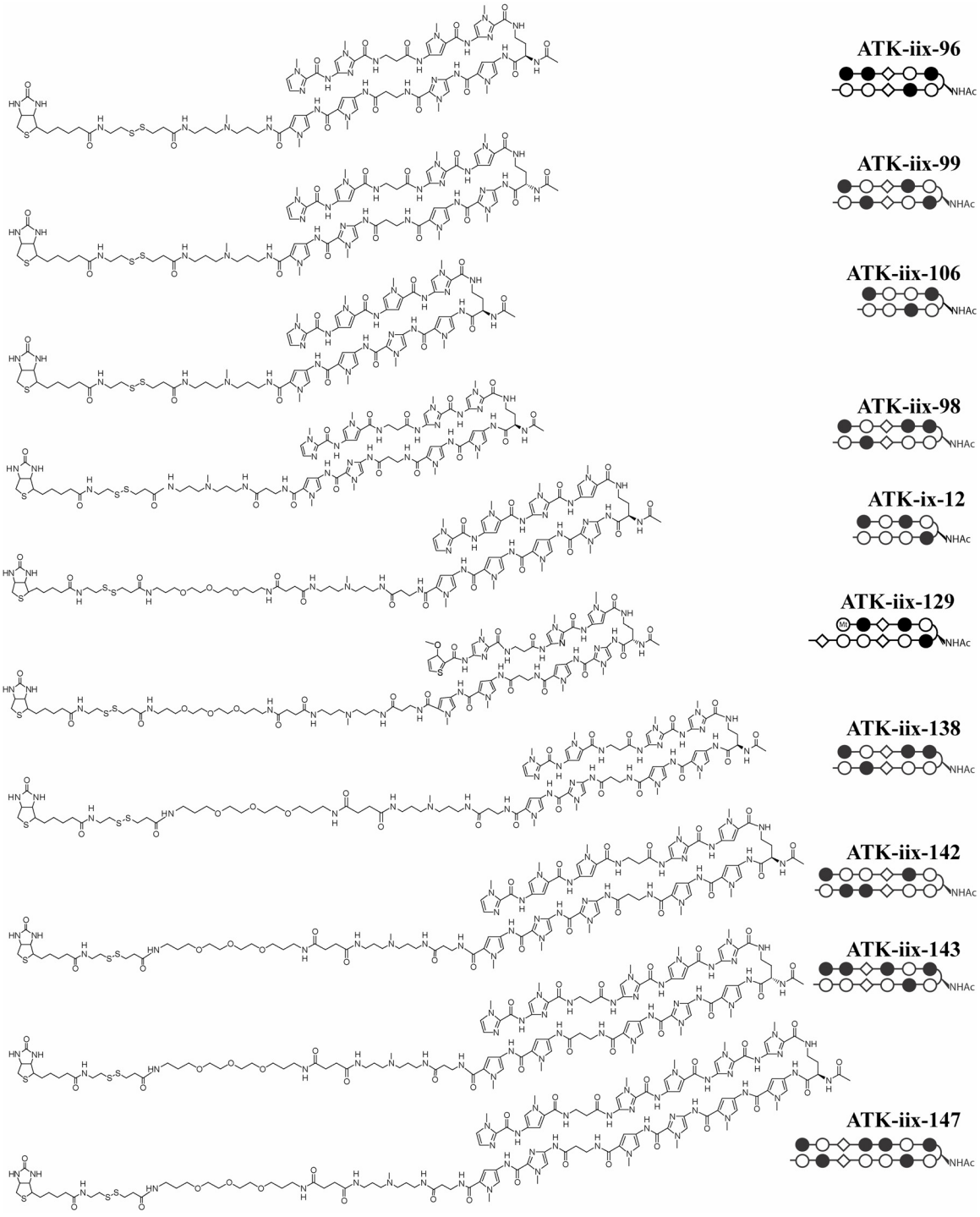


Figure I.9. Chemical structures of the hairpin polyamide-biotin conjugates synthesized for pUC19 pulldown studies (left column). Shown at right are the ball-and-stick representations of the polyamide cores for each of the conjugates. Compound names are also shown at right.

beads were then added, and the mixture shaken for 15 minutes. The beads were washed, and the DNA released by treatment with 50 mM DTT for 30 minutes. Both the supernatant (from the pulldown reaction) and the release solution were loaded onto a 5% agarose gel and run for 2 hours at 160 volts. The gels were then incubated with the DNA stain SYBR Gold (Molecular Probes) and the gels visualized with the Typhoon PhosphorImager.

A library of polyamide-SS-biotin conjugates was synthesized for these experiments (Figure I.9). The data for these experiments are illustrated in figures I.10-14. Sequence analysis of pUC19 was done to determine the locations of all match sites for each of the polyamides. For most polyamides tested, all the expected fragments were captured and released. In almost every case, the 941 base pair fragment was captured, regardless of the presence of a match site. Pulldown and release yields with these large fragments of DNA were approximately 20%.

In an effort to purify a single fragment from the complex mixture, double pulldown reactions were attempted (Figure I.15). First, DNA was captured and released using either **ATK-iix-89** or **ATK-iix-88**. The DNA in the release solution was then purified by ethanol precipitation to remove the DTT. The resulting DNA was then taken up in TKMC/T20 buffer and incubated with **ATK-iix-99** or **ATK-iix-98**, respectively. A pulldown and release was then performed on this incubation. Each reaction was then resolved by gel electrophoresis. As shown, the 518 base pair fragment is isolated in 2% overall yield in 91% purity.

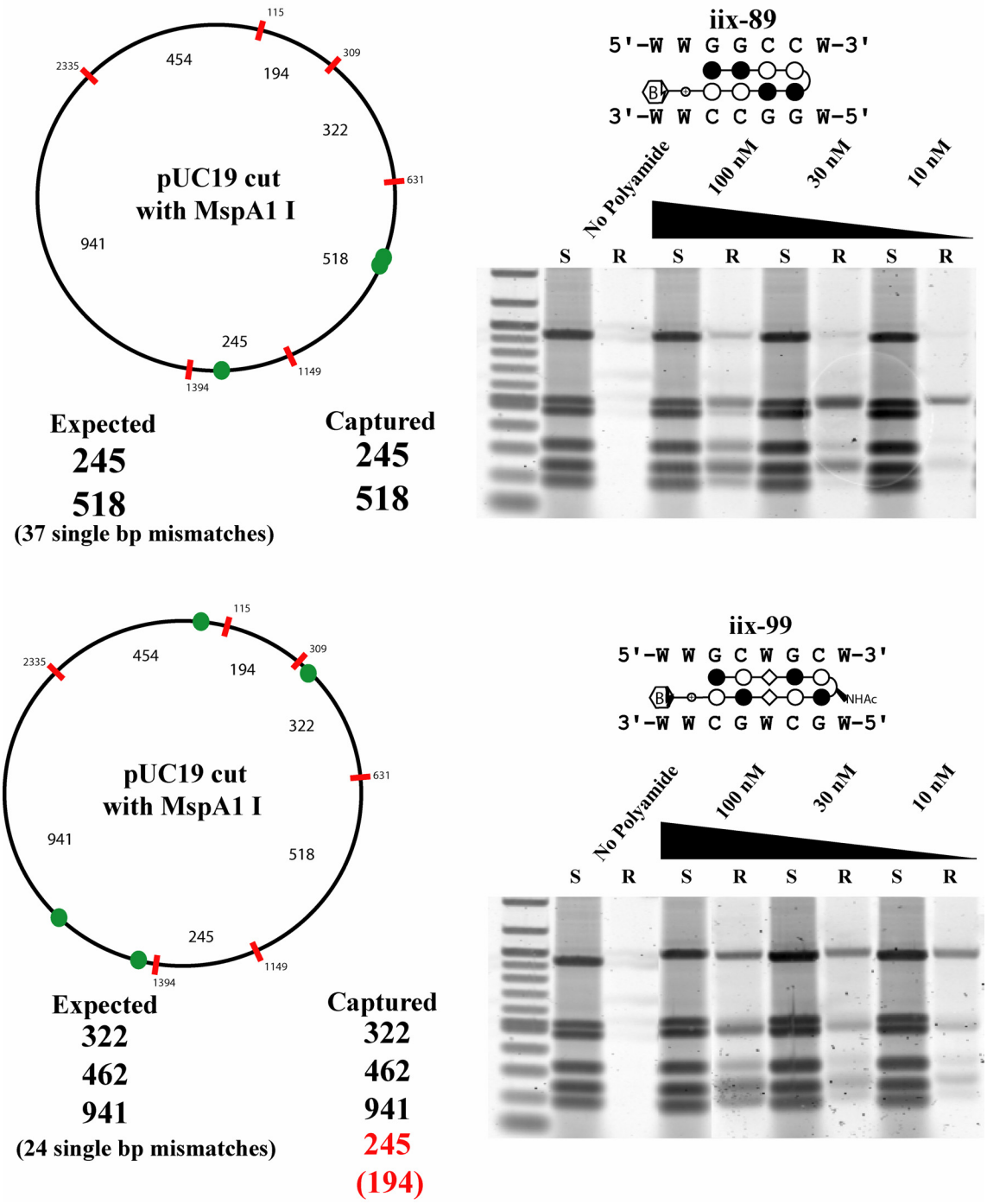


Figure I.10. Pulldown experiments with **ATK-iix-89** and **ATK-iix-99**. Shown at left is a schematic of pUC19 with cut sites (red boxes) and polyamide match sites (green circles). Below left are the expected fragments to be pulled down based on selective match site binding. Below right are the actual fragments pulled down (black = expected; red = unexpected; parentheses = weak). Shown top right is the polyamide with its match sequence. Shown at bottom right is the gel image for the pulldown experiment (S = supernatant; R = release).

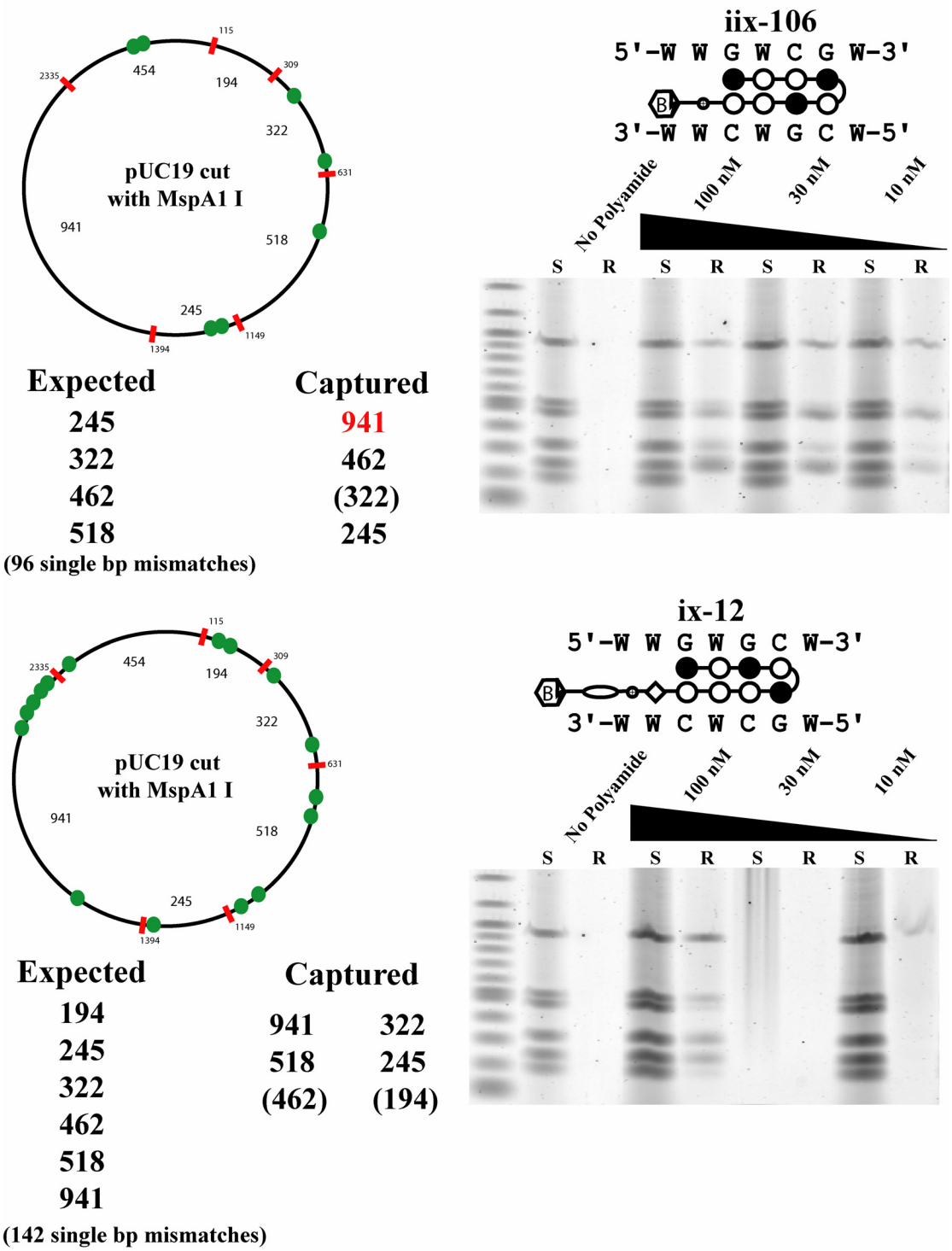


Figure I.11. Pulldown experiments with **ATK-iix-106** and **ATK-ix-12**. Shown at left is a schematic of pUC19 with cut sites (red boxes) and polyamide match sites (green circles). Below left are the expected fragments to be pulled down based on selective match site binding. Below right are the actual fragments pulled down (black = expected; red = unexpected; parentheses = weak). Shown top right is the polyamide with its match sequence. Shown at bottom right is the gel image for the pulldown experiment (S = supernatant; R = release).

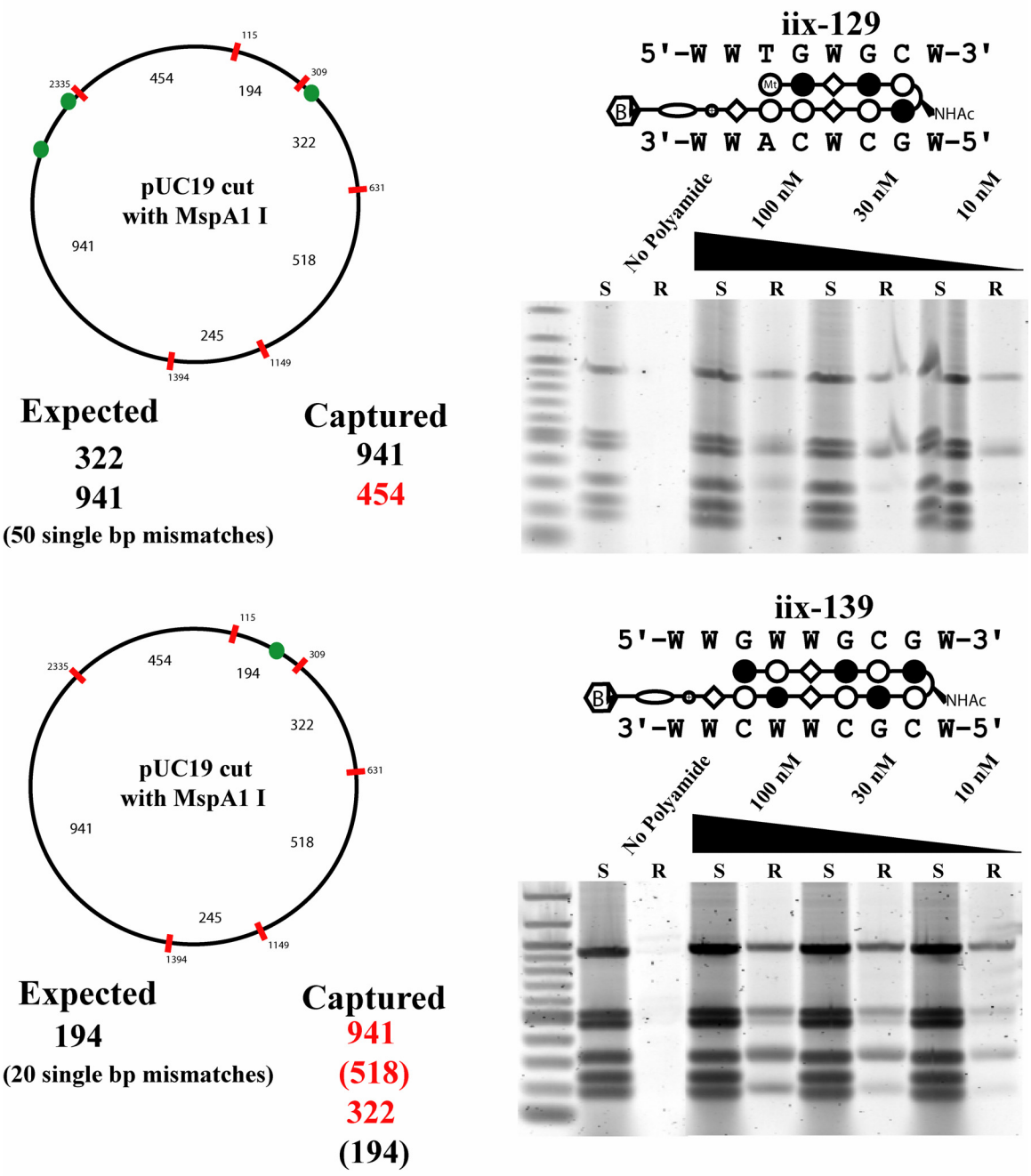


Figure I.12. Pulldown experiments with **ATK-iix-129** and **ATK-iix-139**. Shown at left is a schematic of pUC19 with cut sites (red boxes) and polyamide match sites (green circles). Below left are the expected fragments to be pulled down based on selective match site binding. Below right are the actual fragments pulled down (black = expected; red = unexpected; parentheses = weak). Shown top right is the polyamide with its match sequence. Shown at bottom right is the gel image for the pulldown experiment (S = supernatant; R = release).

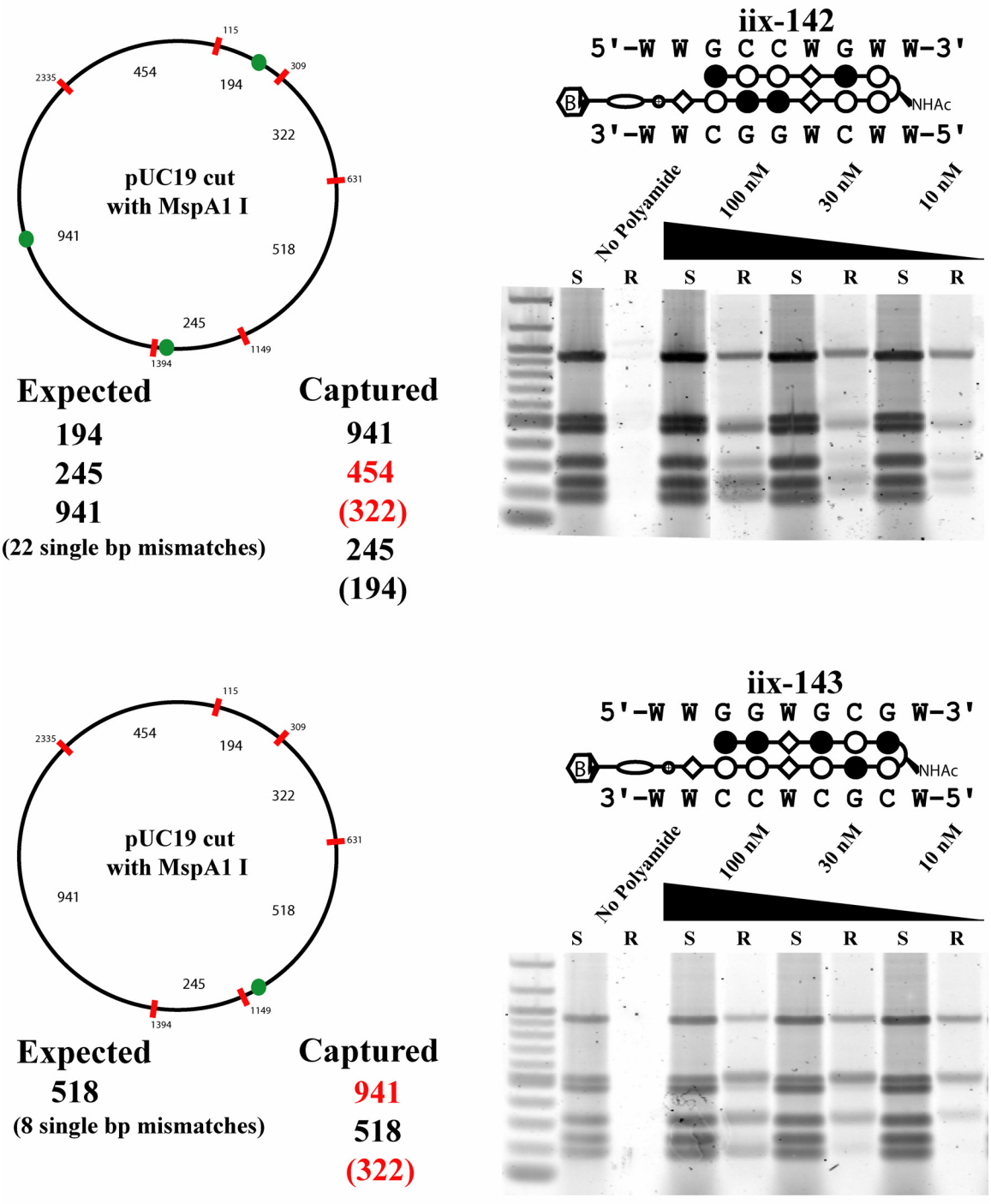


Figure I.13. Pulldown experiments with **ATK-iix-142** and **ATK-iix-143**. Shown at left is a schematic of pUC19 with cut sites (red boxes) and polyamide match sites (green circles). Below left are the expected fragments to be pulled down based on selective match site binding. Below right are the actual fragments pulled down (black = expected; red = unexpected; parentheses = weak). Shown top right is the polyamide with its match sequence. Shown at bottom right is the gel image for the pulldown experiment (S = supernatant; R = release).

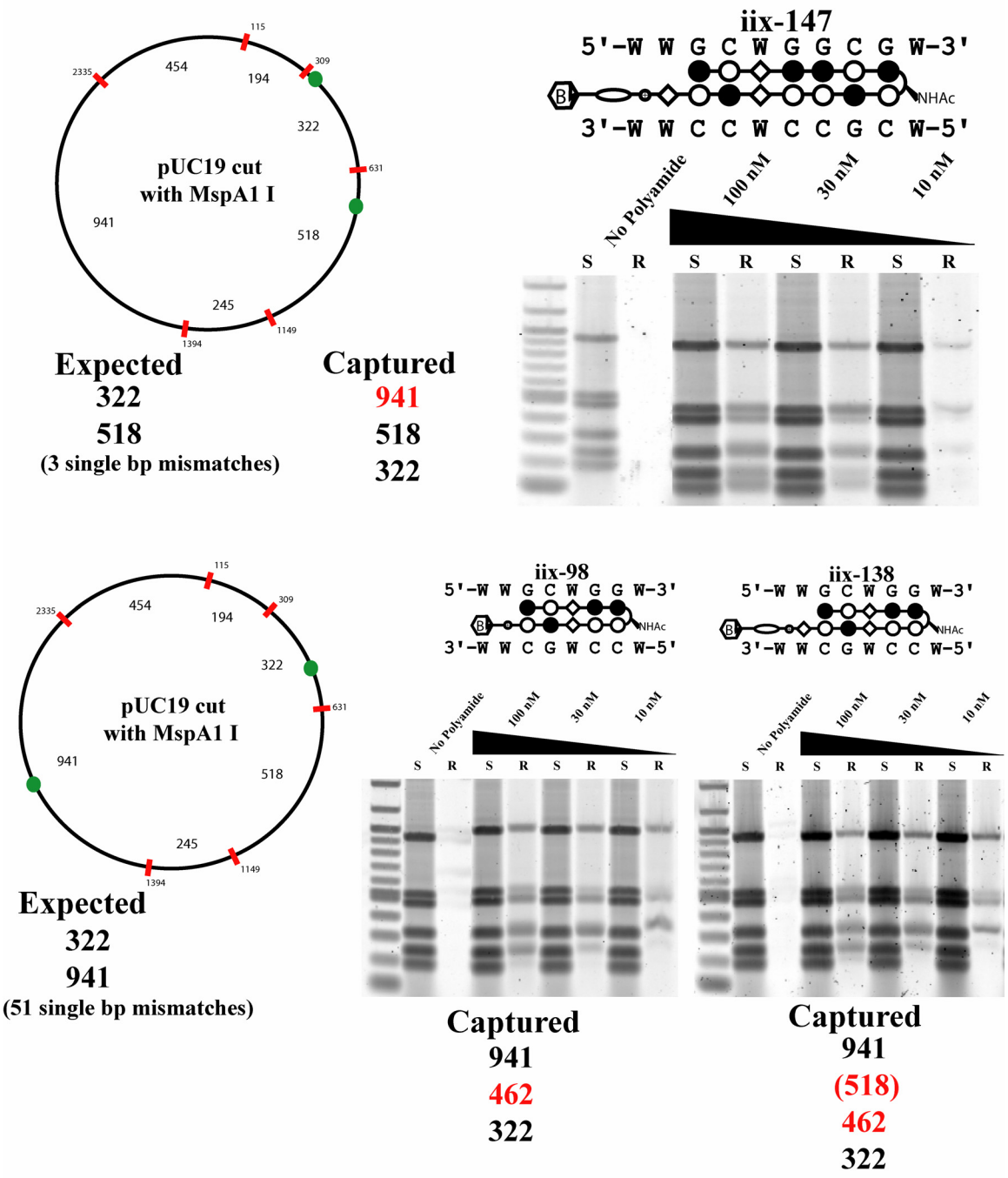


Figure I.14. Top: Pulldown experiments with **ATK-iix-147**. Bottom: Pulldown experiments with **ATK-iix-98** and **ATK-iix-138.** These two polyamides differ only by a PEG linker between the biotin and the polyamide. These data illustrate that the difference in linker length does not affect pulldown specificity. Shown at left is a schematic of pUC19 with cut sites (red boxes) and polyamide match sites (green circles). Below left are the expected fragments to be pulled down based on selective match site binding. Below right are the actual fragments pulled down (black = expected; red = unexpected; parentheses = weak). Shown top right is the polyamide with its match sequence. Shown at bottom right is the gel image for the pulldown experiment (S = supernatant; R = release).

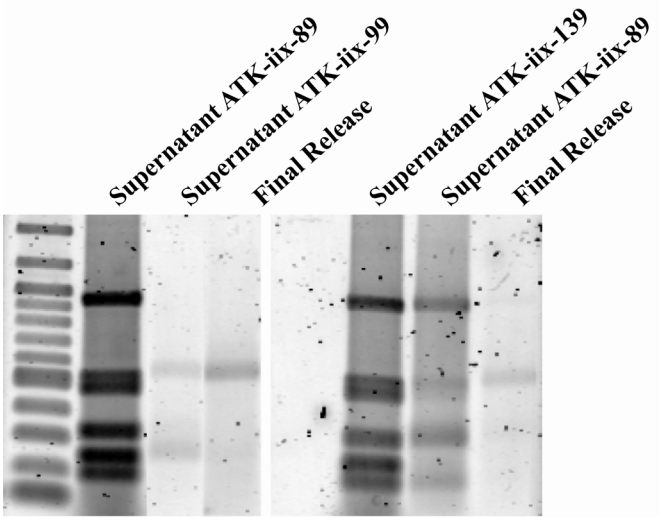


Figure I.15. Double pulldown experiments able to isolate a single DNA fragment from a complex mixture of pUC19 digestion fragments. Leftmost lane contains the DNA size ladder. Next 3 lanes- First double pulldown experiment- supernatant from 1st pulldown with ATK-iix-89; supernatant from 2nd pulldown (performed on the "release" fraction from pulldown 1) with ATK-iix-99; final release. Rightmost three lanes- Second double pulldown experiment- supernatant from 1st pulldown with ATK-iix-139; supernatant from 2nd pulldown (performed on the "release" fraction from pulldown 1); final release.

Ars1

Our collaborator (Michael Foulk; graduate student in the Susan Gerbi lab at Brown University) are interested in the autonomous replicating sequence 1 (ARS1) in the yeast genome (Figure I.16).[14] This site is one of the major origins of replications for the yeast genome, and full characterization of this 250 base pair region of DNA would be of great value to the field. The ARS1 sequence, as well as many of the factors bound to it

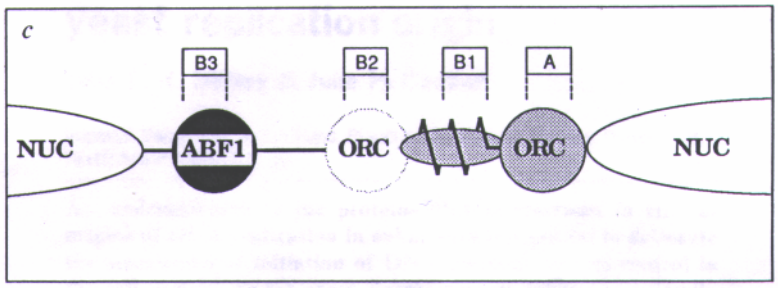


Figure I.16.[14] A plausible molecular model to explain the results of DNase I genomic footprinting at the yeast ARS1 replication start site. The origin recognition complex (ORC) is known to bind to areas A and B1. Transcription factor ABF1 binds to B3. The 250 base pair region is flanked by two tightly positioned nucleosomes.

are well-known. Using polyamide-biotin conjugates, our collaborators hope to be able to isolate the 500 base pair restriction fragment containing the ARS1 sequence (Figure I.17) in order to fully characterize all associated proteins. Polyamides have been designed for this sequence (shipped to Brown University on February 11, 2004).

Figure I.17. Sequence of the 500 base pair restriction fragment containing the 250 base pair ARS1 sequence from the yeast genome. The sequence is listed 5' → 3'. The red underlined section is the region between B2 and B3 (Figure I.16) and should be most accessible to polyamides. Also shown are the polyamides designed to target this sequence (sent to the Gerbi lab, at Brown University, 2004).

Discussion.

These experiments demonstrate that fragments of DNA can be bound and isolated using polyamide-SS-biotin conjugates. Initial pulldown experiments demonstrated that polyamide-biotin conjugates show good specificity for their match sites. Interestingly, **AH-677-2** exhibited far less specificity than **ATK-ix-36**. Perhaps this difference in specificity is a reflection of the fact that the imidazole ring is capable of making a

favorable hydrogen bond with the exocyclic amine of guanine, and **ATK-ix-36** contains more imidazole rings. Because these experiments are done in cell-free conditions, nuclear uptake is not an issue. Thus, larger polyamides capable of targeting long sequences of DNA can be used. In subsequent experiments, an effort was made to design polyamides with high imidazole content in order to maximize specificity. As shown by the pUC19 pulldown experiments, many of the polyamides show significant off-target pulldown. This may be due to the fact that each of the six fragments contains multiple single base pair mismatch binding sites for each of the polyamides tested. Thus, the polyamides may bind and pull down fragments with these multiple mismatches, even when no match site was present. Another issue in moving forward with DNA pulldown on a genomic scale is that as longer pieces of DNA are used, pulldown and release yields drop to under 20%. After two successive pulldowns, isolated yield is only 2%. Thus, in order for this to be feasible in a genomic setting, yields will need to be improved. Perhaps the low yields with the larger fragments of DNA were caused by size exclusion; that is, the larger DNA fragments were not tolerated by the solid support in high concentrations. The use of more magnetic beads with lower streptavidin loading may alleviate this problem. Alternatively, these experiments can possibly take advantage of multivalency. That is, polyamides can be targeted to repeat regions in the DNA. If multiple polyamide-biotin conjugates are present on a single fragment, perhaps that complex's affinity for the streptavidin-coated beads will increase, leading to improved yields. Overall, these experiments represent significant exploratory efforts towards the use of DNA-binding polyamides as tools for molecular biology.

Materials and Methods.

The base polyamides used in this study were synthesized on solid support using previously published methods and reagents.[15, 16] Biotin reagents were purchased from Pierce. Streptavidin-coated magnetic beads are from Dynal. Fluorescent oligonucleotides were purchased from IDT DNA. pUC19 was purchased from Sigma. The SYBR Gold stain was purchased from Molecular Probes.

ATK-iix-36. Base polyamide was cleaved from resin using N-methylamino dipropylamine. This was then coupled to the biotin using PFB-PEO-Biotin. UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1625.8. Found 1625.3.

ATK-iix-26. Synthesized according to literature procedures.[13] UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1946.8. Found 1946.8.

ATK-iix-47. Synthesized from base polyamide using NHS-iminobiotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1724.6. Found 1724.9.

ATK-iix-73. Synthesized from base polyamide using NHS-desthiobiotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1694.7. Found 1694.9.

ATK-iix-89. Synthesized from base polyamide using SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1586.4. Found 1586.5.

ATK-iix-94. Synthesized from base polyamide using SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1584.5. Found 1584.8.

ATK-iix-98. Synthesized from base polyamide using SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1786.4. Found 1786.1.

ATK-iix-99. Synthesized from base polyamide using SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1785.2. Found 1785.2.

ATK-iix-106. Synthesized from base polyamide using SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1713.9. Found 1713.5.

ATK-iix-33 (**Mono Boc-protected 1,14-(5,8,11-trioxa)undecane diamine)**. 1,14-(5,8,11-trioxa)undecane diamine (10 g, 58.1 mmol) was cooled to 0 ºC. Boc anhydride (1.58 g, 7.26 mmol) in 20 mL DCM was added drop wise over 30 minutes. The reaction was warmed to rt, and stirred for 1 h. 80 mL of ¼ saturated $NaHCO_3$ was then added, and the product extracted into ethyl acetate (3 x 50 mL). The combined organics were washed with saturated $NaHCO_3$ (2 x 50 mL) and brine (2 x 50 mL), dried over anhydrous magnesium sulfate, and concentrated by rotary evaporation. Product was isolated as a clear oil (2.19 g, 100%). ESI Mass [M + H] calcd. 320.4. Found 320.4.

ATK-iix-34 (BocNH-(5,8,11-trioxa)undecane succinic acid). ATK-iix-33 (2.1 g, 7.3 mmol) was dissolved in 20 mL DCM. DIEA (1.2 g, 9.6 mmol) was added and the reaction stirred for 5 minutes. Succinic anhydride (861 mg, 8.3 mmol) was then added and the reaction was stirred at room temperature overnight. The reaction was rotovapped to dryness, and the product purified by flash chromatography (10% MeOH:$CHCl_3$, rf = 0.7). Product was isolated as a white solid (1.8 g, 66%). ESI Mass [M - H]+ calcd. 419.5. Found 419.8.

ATK-iix-129. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 2193.8. Found 2193.7.

ATK-iix-138. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 2158.8. Found 2158.4.

ATK-iix-139. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (85900). MALDI-TOF-MS calcd. (M + H): 2402.8. Found 2402.5.

ATK-iix-142. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (85900). MALDI-TOF-MS calcd. (M + H): 2402.8. Found 2402.4.

ATK-iix-143. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (85900). MALDI-TOF-MS calcd. (M + H): 2403.6. Found 2403.9.

ATK-iix-147. Synthesized from base polyamide, first coupling **ATK-iix-34** (1.5 equiv), PyBOP (5 equiv.), DIEA:DMF (4:1, 300 μL/μmol), followed by deprotection with 50% TFA:DCM, and final coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (1030800). MALDI-TOF-MS calcd. (M + H): 2648.5. Found 2648.7.

ATK-ix-8. Synthesized from base polyamide by coupling with SulfoNHS-SS-Biotin (Pierce). UV (H_2O) λ_{max} 310 nm (68720). MALDI-TOF-MS calcd. (M + H): 1388.5. Found 1388.2.

Sample Pulldown and Release Protocol.

Prepare:

5 x TKMC/T20 Buffer:
- 50 mM Tris-HCl
- 50 mM KCl
- 50 mM $MgCl_2$
- 50 mM $CaCl_2$
- pH = 7.0
- 0.2 uM Filter
- ***Add Tween20 to the completed buffer to 0.5%***

10x Solution of Polyamides in Water

50 mM DTT / 0.1% Tween 20 in Water

PROCEDURE:

1) Reactions are performed in 40 uL reaction volumes if doing one pulldown, 200 uL reaction volumes if doing two pulldowns.

2) Set up the Polyamide/DNA equilibration (15 nM [DNA], 10-100 nM [PA])

- 4 uL 10x PA solution
- 8 uL 5x TKMC/T20 solution
- x uL DNA solution in water (to 15 nM)
- 28-x uL H_2O
- 40 uL total volume

3) Vortex to mix (1–2 sec), QuickSpin on benchtop centrifuge and place tubes in a still, dark place to equilibrate for 3–14 hours.

4) After equilibration, set up pulldown tubes, these are new tubes with dry streptavidin-coated beads in them. Use 100 μg beads for every 50 μL of a 50 nM solution of polyamide. If you are far below this amount, use 50 μg of beads (that is, never use less than 50 μg beads). To prepare the beads:

- Take the appropriate amount of beads out of the stock solution for the total number of reactions.

- Dilute to 1 mL with 1x TKMC/T20.
-Using a magnet, trap the beads and remove the supernatant.
-Fill the tube with 1 mL 1x TKMC/T20.
-Remove supernatant again.
-Repeat the washing steps once more, then dilute to 1 μg/μL with 1x TKMC/T20.
-Transfer the appropriate amount of bead solution to each pulldown reaction tube (make sure that the beads are well suspended in the liquid (i.e., do not centrifuge before aliquoting).

5) Remove supernatant and add the appropriate DNA/PA solution to the washed and dried beads.

*** Be careful not to leave the beads out of solution for too long as they will not work after this.***

6) Briefly vortex the pulldown tubes to mix, centrifuge, and then set on a shaker for 20–60 min to equilibrate the biotin/streptavidin.

*** It is important in this step for the beads to remain suspended in the solution. A VWR Thermomixer shaking heat block set to 1400 rpm will accomplish this. An interval that shakes for 10 seconds and then rests for 1 min 50 seconds was found to be optimal. ***

7) When complete, centrifuge, let rest for 3–5 minutes, then, using the magnet, capture the beads and remove the supernatant into new tubes. ***This is the SUPERNATANT lane for gels. ***

8) Wash the dry beads with 40 μL 1x TKMC/T20, very briefly vortex, spin, and quickly remove the supernatant (this wash may be discarded).

9) Add 40 μL of the 1x DTT/T20 solution to the dry, washed beads and shake for 30 min-1 hour at 37 ºC using the same interval mix as in step 6.

10) When done, vortex, spin, capture beads with magnet, and remove the supernatant *** This is the RELEASE lane for gels.***

11) If you are only doing a single pulldown, skip to step 13; If you are doing multiple pulldowns proceed to step 12.

12) Take the RELEASE tubes and do a phenol:chloroform extraction, keeping the aqueous layer.

13) To all tubes (SUPERNATANT and RELEASE) add 2 volumes EtOH, invert to mix, and spin at 14000 rpm at 4 ºC for 30 minutes to precipitate the DNA.

14) Remove the supernatant. If doing multiple pulldowns, skip to step 17.

15) Let air dry for 15 min, then take up in 20 μL H_20. Dissolve the pellet by vortexing (5 sec), spinning, then letting the tubes sit for 15 min.

16) Add 5 uL 10x Ficol/TBE (to final conc. of 2x) and load on an agarose gel. Run gel. Visualize with SYBR Gold stain using Typhoon Phosphorimager.

For multiple pulldowns

17) To the RELEASE tubes from step 10 (carried through steps 11–14) Let the tubes air dry 15 minutes then take up the pellet in a new solution of 1 x TKMC/T20 and polyamide. Because of the low pulldown yield, I usually use the same concentration of polyamide as I did in the first pulldown reaction but I decrease the volume by ~4-fold. This solution then becomes your second equilibration reaction. It can then be carried through steps 3 to the end.

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