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Automated Selection of Anti-Protein Aptamers

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Abstract—The in vitro selection of nucleic acid binding species (aptamers) is frequently repetitive, time-consuming, and poorly adapted to high-throughput applications. We have adapted automated workstations to select anti-protein aptamers; as an example, we demonstrated the selection of anti-lysozyme aptamers that function as efficient inhibitors of cell lysis. The increases in throughput brought about by automation should potentiate the application of aptamer technology to the rapidly growing field of proteomics. © 2001 Published by Elsevier Science Ltd.

Introduction

In order to develop sensor arrays that can be used to probe and analyze large molecular mixtures, such as organismal proteomes and metabolomes, it may be necessary to generate affinity reagents that can recognize each protein or metabolite in the mixture. The proven pluripotent binding abilities of antibodies makes them an obvious choice for the development of arrays.[1–3] However, the selection of antibodies from phage- or cell-surface displayed libraries still requires bacterial transformation, ultimately limiting the high-throughput applications of these methods.

The only other affinity reagent that may be capable of recognizing the diverse targets in proteomes and metabolomes are nucleic acid binding species (aptamers). Aptamers have been selected against a variety of targets, ranging from small organic molecules[4–6] up to supramolecular structures, including organisms.[7,8] More importantly, we have previously shown that it may be possible to automate nucleic acid selection methods.[9] We now demonstrate the automated selection of anti-protein aptamers, and suggest automated methods for acquiring receptors for organismal proteomes.

Materials and Methods

Target and pool

Lysozyme purified from hen egg white was purchased from Sigma-Aldrich (St. Louis, MO). The enzyme was suspended at a 1 mg/mL concentration in the selection buffer (see below), and chemically biotinylated using sulfo-NHS-LC-biotin (Pierce, Rockford, IL). The biotinylation reaction occurred at a 10× molar ratio of biotinylating reagent to enzyme, and proceeded for 2 h on ice. Unincorporated biotin was removed via a 10DG chromatography desalting column (Bio-Rad, Hercules, CA). The biotinylated lysozyme was then captured by magnetic strepavidin-coated Dynabeads (Dynal, Lake Success, NY). The nucleic acid library used for selection, N30, has been previously described.[10,11]

Automated in vitro selection. Exact volume displacements and robotic manipulations are as previously described.[9] Significant changes to the original protocol are discussed in Results and Discussion.

Robotic workstation configuration. The entire selection was carried out on a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA), shown in Figure 1. Control of the robot was governed directly by Bioworks 3.1c (Beckman Coulter). The robot's worksurface is integrated with a PTC-200 thermal cycler (MJ Research, Waltham, MA) in a remote dock position, a MPC auto-96 magnetic particle separator (Dynal), a modified Multiscreen vacuum filtration manifold (Millipore, Bedford, MA), and a 'homemade' enzyme cooler. The enzyme cooler exchanges heat via a recirculating liquid bath and is capable of holding 96-wells of enzyme solution at −20 °C indefinitely. A Stacker Carousel (Beckman Coulter) funnels fresh pipette tips to the workstation as needed.

Automated partitioning of binding species. At the start of a round of the selection, the workstation pipetted RNA

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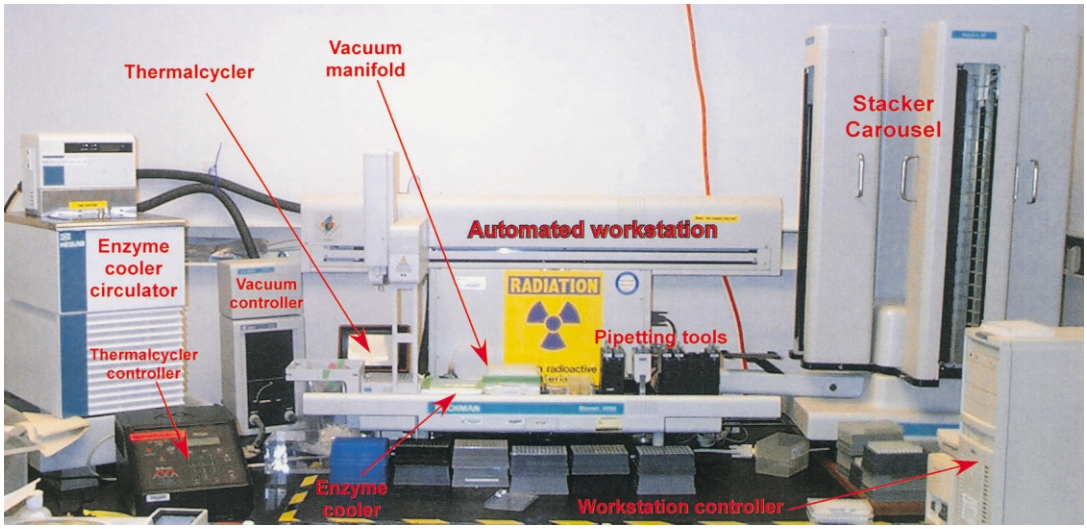

Figure 1. Selection robot. A photograph of the entire automated in vitro selection system. It consists of a Beckman Coulter Biomek 2000 robot, integrated with a thermal cycler, magnetic particle separator, vacuum filtration manifold, pipette tip carousel, and enzyme cooler. A personal computer controls all the components.

pool to 400 mg of Dynabeads with biotinylated lysozyme bound to the beads' strepavidin-derivitized surface. In the first round, the amount of RNA pool applied was 5 μg (ca. 1.1×10^{14} sequences). At all other rounds, the amount of RNA pool applied was one-fifth of the preceding RNA transcription reaction (20 μL). In general, this meant approximately one to three micrograms of RNA pool, but values likely varied between rounds. The RNA pool was dispensed directly onto the beads equilibrated in selection buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$; 80 μL). The RNA pool and bead mixture (100 μL) was incubated for 3 min, thoroughly mixed by pipette aspiration, and incubated for a further 3 min.

After incubation, the bead and RNA mixture was resuspended by mixing, and transferred to the Multiscreen vacuum manifold containing a Millipore HV (PVDF) filter plate. The beads were placed on the membrane surface, and the liquid was siphoned through the membrane. Non-binding RNA species were partitioned away from the bound RNA associated with the lysozyme by vacuum filtration. The beads were then washed with 250 μL of selection buffer, and resuspended on the filter plate in another 250 μL without vacuum. That solution was again filtered, and the wash process proceeded once more for a total of two bead resuspensions in 1 mL of wash buffer.

Automated RT–PCR cycling. The RNA bound to the washed beads was used as a template for a RT–PCR reaction. After washing, the beads were suspended in pure water, and transferred to the thermal cycler (51 μL). Binding species were eluted from their targets by holding the mixture at high temperature (98 °C) for 3 min. Afterwards, 45 μL RT–PCR reaction buffer (final concentrations were 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% acetamide, 0.05% Nonidet P40, and 0.5 μM each of 5′ and 3′ primers) was added. If the amplified DNA was to be radiolabeled, 5 μCi of α-^{32}P-labeled dATP was also added to the reaction.

After addition of reaction buffer, the mixture was incubated at 65 °C for 10 min to disrupt the structures of selected RNAs. The RT–PCR enzyme mixture (4 μL) was then pipetted from the enzyme cooler. The mixture contained 5 U of AMV reverse transcriptase (Amersham Pharmacia Biotech, Arlington Heights, IL), and 0.2 U of Display Taq (Display Systems, Vista, CA). To minimize the high pipetting error associated with the pipette transfer of small amounts of 50% glycerol solutions (>1 μL), the enzymes are diluted together in a solution of 50% glycerol, 10 mM Tris (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$ prior to the selection run. The enzyme mixture was mixed with the RT–PCR reaction mixture by pipette aspiration.

Next, the automated lid on the thermal cycler closed and warmed to 110 °C. Reverse transcription was allowed to proceed for 10 min at 50 °C, at which time PCR cycling began. The amplification reaction was cycled (45 s at 94 °C, 60 s at 50 °C, and 90 s at 72 °C) either for a total of 20 cycles (selection rounds 1–6) or 16 cycles (selection rounds 7–12). After cycling was complete, 10% of the reaction was mixed with an equal volume of stop dye for future gel analysis, and 50% of the reaction was placed in a separate microplate for archival purposes.

Automated in vitro transcription. 10% of the RT–PCR mixture (10 μL) was used as template to generate RNA for the next round of selection. The DNA template was mixed with 87 μL RNA transcription buffer (final concentrations were 40 mM Tris (pH 7.9), 26 mM $MgCl_2$, 5 mM dithiothreitol, and 2.5 mM of each NTP). If the RNA produced was to be radiolabeled, 5 μCi of α-^{32}P-labeled UTP was also added to the reaction. After

mixing, 40 U of RNasin (Promega, Madison, WI) ribonuclease inhibitor and 100 U of T7 RNA polymerase (Stratagene, La Jolla, CA) were added and well-mixed. The final volume of the reaction was 100 μL and the reaction proceeded for 75 min at 37 °C. At the conclusion of the reaction, 10% was mixed with an equal volume of stop dye for gel analysis.

Additional automated selection rounds. 20% of the transcription reaction (20 μL) was added to a new well containing 400 mg of lysozyme-covered beads to start the next round of selection. For this selection, 12 rounds of selection were executed, requiring less than two days time (~42 h).

Characterization of clones

DNA generated from the automated selection was ligated into a thymidine-overhang vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA). Individual transformants were grown overnight in LB, and DNA isolated using a Plasmid Mini Kit (Qiagen, Valencia, CA). To obtain the sequence of aptamers, the plasmids were cycle-sequenced using a Sequitherm Excel II DNA Sequencing Kit (Epicentre, Madison, WI).

Individual aptamers were assayed for binding activity as previously described[12] using a Minifold I filtration manifold (Schleicher & Schuell, Keene, NH) that sandwiched a Protran pure nitrocellulose membrane (Schleicher & Schuell) and a Hybond-N+ nylon transfer membrane (Amersham Pharmacia Biotech). Binding reactions were filtered through the filtration manifold and washed with a high salt solution (20 mM Tris (pH 7.5), 1 M NaCl, 5 mM $MgCl_2$) to disrupt nonspecific electrostatic interactions between the positively charged lysozyme molecules and negative phosphate backbone of the RNA molecules.[12,13] Percentages of bound nucleic acids were computed using a PhosphorImager SI (Amersham Pharmacia Biotech). Dissociation constants were determined by assaying the binding ability of limiting amounts of aptamer over a wide range of protein concentrations (6.8 pM–1.3 μM). The log of protein concentrations versus the amount of aptamer bound was fit to the equation $y = a \times x/(b + x)$ and directly yielded K_d values.

Lysozyme activity assay

To evaluate the effect of aptamers on the function of lysozyme in vitro, lysozyme activity was assayed using a standard method.[14,15] In short, lyophilized *Micrococcus lysodeikticus* (ATCC No. 4698) cells (Sigma-Aldrich) were suspended in selection buffer at an O.D. (450 nm) of about 0.9. An aliquot (250 μL) of suspended cells was placed in a microplate tray in which either nothing, lysozyme, RNA, or combination of lysozyme and RNA was added, for a total volume of 300 μL. Wells with enzyme received 1 μg lysozyme, and RNA was added at a 10× molar ratio to that of lysozyme. Assay wells were measured for their absorbance at 450 nm for 1 h in real-time in a FL600 microplate reader (Bio-Tek Instruments, Winooski, VT).

Results and Discussion

Development of an automated selection protocol

We had previously developed an automated protocol for the in vitro selection of nucleic acids that bound to oligonucleotide targets.[9] This protocol was also the starting point for the automated selection of anti-protein aptamers (Fig. 2), but required several significant modifications. The most important modification was that filtration was used for the actual selection step, rather than magnetic bead capture. As has previously been observed for manual selections,[16] filtration more efficiently separates protein/nucleic acid complexes from free nucleic acids than does magnetic bead capture. It was hoped that this more stringent selection method would yield aptamers with affinities similar to those observed in manual selections (typically nanomolar K_d values). In order to carry out filtration on the Biomek 2000, it was necessary to adapt a commercially available vacuum manifold system (Millipore Multiscreen) to function in a defined space on the worksurface of the selection robot.

It initially proved difficult to adapt filtration selections to the robot, primarily because the Biomek could not mechanically rend the filter and because the nitrocellulose filters have an intrinsic affinity for proteins. To overcome these problems we developed a special form of filtration that could be easily adapted to the robot: targets were biotinylated and immobilized on streptavidin beads, and the beads were subsequently filtered on a PVDF membrane with low protein-binding capacity. This method has the advantage of being less susceptible to the accumulation of filter-binding species than are manual filter-based selections.

Several changes were made in the amplification procedure in order to efficiently recover the highest affinity aptamers. Previously, bound nucleic acid species were eluted from their oligonucleotide targets by heating the magnetic beads in the PCR machine on the robot, transferring the beads to a magnetic microplate device, and pipetting off the supernatant. Unfortunately, during the ~45 s that these manipulations take, the heated bead solution typically cooled to the point that some of the highest affinity RNA species rebound to the target. To obviate this problem, we simply elute bound RNAs directly from the beads during the initial heating steps of RT–PCR reactions. The direct amplification of bead-bound nucleic acids has previously been successfully demonstrated.[17,18] In order to avoid the over-amplification of selected species, we also progressively reduced the number of thermal cycles that were carried out during the course of the selection. We have empirically determined that at the beginning of selection experiments 20 thermal cycles will typically yield enough template for in vitro transcription reactions, while near the conclusion of the selection (10 rounds of selection or more) only 14–16 cycles are necessary.

A number of more minor but nonetheless important changes in method were also required to adapt the automated selection protocol to proteins. For example,

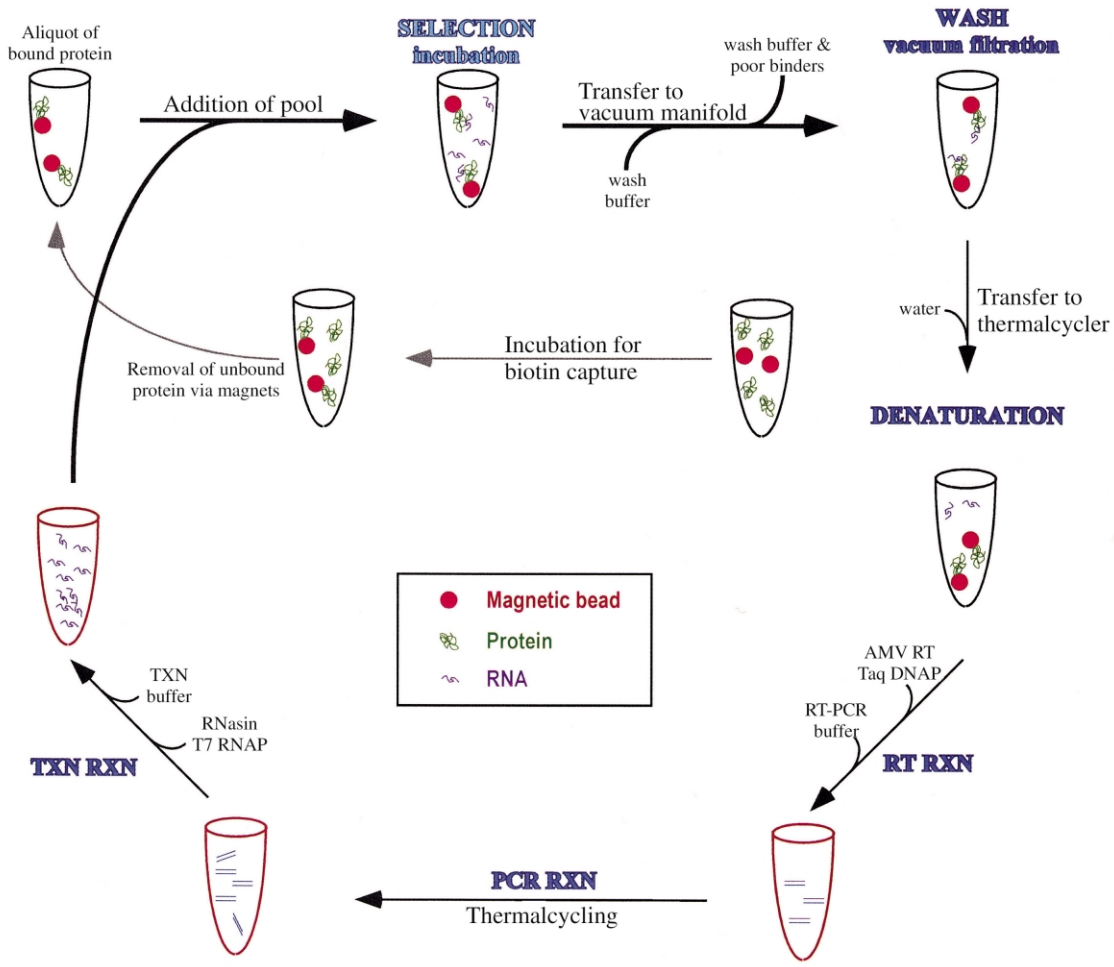

Figure 2. Selection of anti-lysozyme aptamers. A simplified schematic depicting a round of automated in vitro selection. Magnetic beads derivatized with streptavidin are shown as red balls, biotinylated protein targets are shown in green, and the RNA pool is purple. Liquid manipulations are shown in black tubes, while enzymatic amplification steps are shown in red tubes. A typical round of selection involves adding the RNA pool to a protein target captured on magnetic beads, filtration washing of the beads, elution of selected binding species, and amplification to regenerate RNA for the start of the next round.

the original in vitro transcription buffer contained not only the components listed in the Materials and Methods section, but also 0.01% Triton X-100 and 2.5 mM spermadine. These two ingredients were omitted from the reaction buffer in the anti-protein selection experiments as it was determined that the presence of these chemicals—especially the highly-charged polyamine spermadine—interfered with the ability of the nucleic acid pool to interact with the target protein. Normally, this is not an obstacle when doing manual selections, since the transcription reaction is typically gel purified.

Performance of the automated selection protocol

The automated protocol was initially proofed by allowing the robot to complete the final rounds of a manual selection experiment. The tyrosyl tRNA synthetase from Neurospora mitochondria (Cyt18) is known to bind Group I introns with extremely high affinity.[19,20] Ten rounds of manual selection were first carried out against Cyt18, and an additional ten rounds of selection were then carried out on the Biomek 2000. The results of these selection experiments will be reported elsewhere (Cox and Ellington, in preparation), but they revealed that the robot could adequately carry out all of the necessary manipulations for an in vitro selection experiment, could produce adequate amounts of double-stranded DNA for in vitro transcription and adequate amounts of RNA for selection, and could winnow high affinity species from the population. While some high molecular weight PCR products accumulated during the final rounds of the selection, these products did not interfere with the eventual isolation of high affinity aptamers.

Automated selection of anti-protein aptamers

Based on these results, we attempted a fully automated selection of anti-protein aptamers. Our initial target was a protein not normally known for its nucleic acid binding abilities, hen egg white lysozyme (EC 3.2.1.17). We chose this protein because a very pure preparation was available commercially. The protein was also known for its exceptional stability. Finally, sequence analysis of this protein revealed the presence of several basic patches that might prove favorable for aptamer selection.

(a)

Clone 1. ATCAGGGCTAAAGAGTGCAGAGTTACTTAG
20/33 clones = 61% of sequences

Clone 2. GGTGATCATGGCAGTGTACGCGGGCGGACA
4/33 clones = 12% of sequences

Clone 3. GGTTGTGAAGATTGGGAGCGTCGTGGCTAC
3/33 clones = 9% of sequences

Clone 4. GTAAATCGTCGACAGGAATTGGCGGGCCGG
3/33 clones = 9% of sequences

Clone 5. GAATTGCGACAGTCGGGACATGTCGCGAGG
2/33 clones = 6% of sequences

Clone 6. GGAATGAGTGCCCTGCAAGCGAGGGCTAGC
1/33 clones = 3% of sequences

(b)

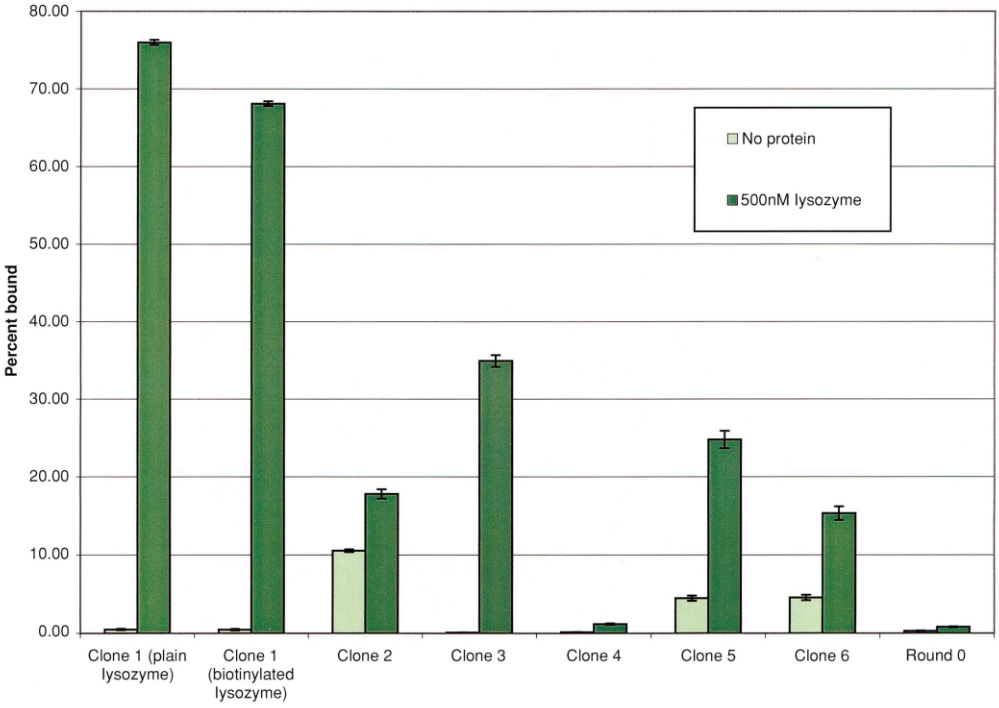

Figure 3. Sequences and activities of anti-lysozyme aptamers. (a) The clones remaining after 12 rounds of selection. Only the random region (30 nt) is shown. (b) The binding activities for the selected aptamers are shown. Light green bars illustrate percent binding in the absence of protein, while dark green bars show percent binding in the presence of 500 nM lysozyme.

Twelve rounds of automated selection were carried out as described above and in Materials and Methods. While the nascent population actually showed a reasonable affinity for lysozyme under the buffer conditions used for the selection (19.6% binding), under more stringent assay conditions virtually none of the original pool bound (0.8%), while 68.1% of the selected population bound. Although a large fraction of the original pool was weakly bound, the lysozyme target was limiting relative to the RNA pool throughout the selection, ensuring the identification of high-affinity aptamers. Individual aptamers were cloned and sequenced. Sequence comparisons revealed that only six individual binding species remained in the population (Fig. 3a). One of these individuals comprised 61% of the population. Given that the starting population was estimated to contain 1.1×10^{14} members, this indicates an enrichment over the starting pool of roughly 10^{12}-fold.

Clone 1, the most populous individual, also proved to be the tightest binding aptamer in a standard binding assay (Fig. 3b), although all but one of the clones (Clone 4) bound to lysozyme much better than the starting pool. In addition, only one of the aptamers (Clone 2) showed significant binding in the absence of protein, indicating that our safeguards against the accumulation of filter-binding sequences were largely successful. Despite the fact that the selection target was biotinylated lysozyme, the aptamer actually binds slightly better to unbiotinylated protein. The aptamer formed a complex with biotinylated lysozyme that had a dissociation constant of 65 nM, while the same aptamer

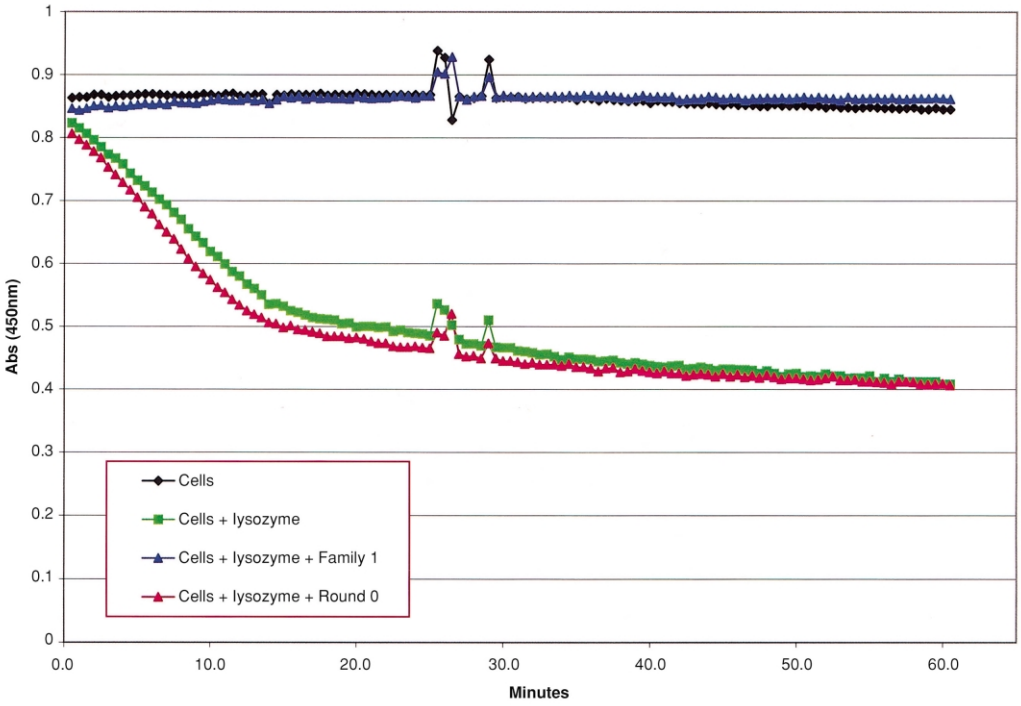

Figure 4. Lysozyme inhibition by aptamer graph. The cytolytic effect of lysozyme on bacterial cells is plotted as a function of the decrease of a cell culture over time. Cells (black) maintain their absorbance while cells incubated with lysozyme lose absorbance (green). Cells that are incubated with lysozyme and the Clone 1 aptamer (blue) demonstrate no discernable decrease in absorbance while cells incubated with the same amount of unselected RNA (red) are efficiently lysed.

formed a complex with unbiotinylated lysozyme that had a dissociation constant of 31 nM. The fact that little or no filter- or biotin-dependent binding was observed, bodes well for the eventual application of aptamers isolated by automated selection.

In line with this conjecture, Clone 1 also demonstrated a surprising ability to interfere with native enzymatic function in vitro (Fig. 4). Functional lysozyme destroys the cell walls of many bacteria, causing the cells to lyse. This process can be measured as a decrease in absorbance at 450 nm.[14,15] When a *Micrococcus* culture was incubated with lysozyme a rapid drop in absorbance values was observed. However, when the same reaction was carried out in the presence of a 10-fold excess of Clone 1, no detectable lysis was observed over the course of an hour. The same amount of unselected pool incubated with lysozyme generates no observable hindrance of the rate of lysis. The fact that an aptamer selected for protein-binding also inhibits protein function is consistent with previous observations of a 'homing principle' that leads aptamers to preferentially bind to pockets on proteins, such as active and allosteric sites.[21] Again, these results in general bode well for the eventual application of aptamers isolated by automated selection.

The robotic workstation reported here can carry out eight selections in parallel, and will complete approximately 12 rounds of selection in two days. These figures imply that a single robot could produce aptamers against upwards of 120 targets in a month. This rate of throughput exceeds that of manual selection by a factor of 10 to 100. Although the selection robot can work virtually continuously with only minor pauses for resupply, 120 targets is only a small fraction of those present in an organismal proteome. In order to further increase throughput, we are currently adapting a new generation of a robot, the Biomek FX, to carry out 96 selections in parallel. Assuming that the automated selection procedure remains roughly the same, this platform should give an additional 12-fold increase in throughput, to approximately 1000 or more targets per month. At this rate, several robots working in parallel could potentially develop aptamers against a large fraction of a proteome in a relatively short period of time. However, these estimates should be tempered by the fact that not all targets may be as tractable as lysozyme, and by the possibility that elimination of 'quality control' procedures routinely applied during manual selections, such as size selection of products, may result in the accumulation of artefacts and the failure of some selections.

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