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Isothermal Rolling Circle Amplification of Virus Genomes for Rapid Antigen Detection and Typing

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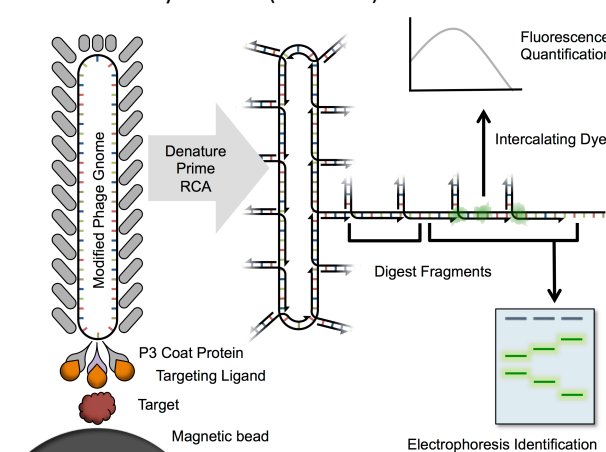
In this work, isothermal rolling circle amplification (RCA) of the multi-kilobase genome of engineered filamentous bacteriophage is used to report the presence and identification of specific protein analytes in solution. First, bacteriophage were chosen as sensing platforms because peptides or antibodies that bind medically relevant targets can be isolated through phage display or expressed as fusions to their p3 and p8 coat proteins. Second, the circular, single-stranded genome contained within the phage serves as a natural large DNA template for a RCA reaction to rapidly generate exponential amounts of double stranded DNA in a single isothermal step that can be easily detected using low-cost fluorescent nucleic acid stains. Amplifying the entire phage genome also provides high detection sensitivities. Furthermore, since the sequence of the viral DNA can be easily modified with multiple restriction enzyme sites, a simple DNA digest can be applied to detect and identify multiple antigens simultaneously. The methods developed here will lead to protein sensors that are highly scalable to produce, can be run without complex biological equipment and do not require the use of multiple antibodies or high-cost fluorescent DNA probes or nucleotides.

Introduction

Since the introduction of immuno-PCR by Sano et al over two decades ago, there has been extensive research into the use of DNA amplification for detecting and quantifying specific proteins in solution.¹ These methods have typically relied on conjugating oligonucleotides to a targeting ligand such as an antibody. Because oligomers with various sequences can be attached to different antibodies and then amplified, this process can be used for multiplex analysis. However, these methods are significantly limited by the length and type of oligonucleotide that can be conjugated to a targeting moiety. It is difficult to attach long end-functionalized DNA strands to antibodies with any appreciable yields and the chemical methods used can also inhibit antibody affinities for specific antigens.²⁻⁴ The need for short DNA strands also limits the number of variously sized templates that can be used in a single assay.^{5,6} A more recent advancement has been the use of isothermal rolling circle amplification (RCA) as a way to create long concatemeric DNA strands that extend from primers attached to detection antibodies.^{7,8} Known as immuno-RCA, this method utilizes small circular ssDNA molecules that anneal to primers attached to detection antibodies and are extended with a strand displacing polymerase. The advantage of this method is that the amplification products remain bound to the detection antibodies and can therefore be used in a microarray format for multiplexed sensing.⁸ However, because the DNA attached to the detection antibody acts as a primer rather than a template, the amplification is linear rather than exponential. Furthermore, as the products are single stranded, their

detection requires the use of either fluorescently labeled complementary oligomers or the incorporation of fluorescent dNTPs, both of which increase the cost of the assay dramatically.^{7,8}

Here, we demonstrate how genetically-engineered filamentous bacteriophage can be used as a multi-kilobase genome reporter for detection of specific protein analytes based on its ability to bind many types of targets and generate inexpensive, multiplexed signals through RCA amplification. First, filamentous phage are micron long viruses that infect bacteria and contain a circular single stranded multi-kilobase genome that codes for the coat proteins on the virus capsid. Filamentous bacteriophage were chosen as sensing platform because peptides and recombinant antibody fragments that bind medically relevant targets can be isolated or expressed as fusions to their p3 and p8 coat proteins through phage display.⁹⁻²² Recently, we also attached detection antibodies to phage by expressing an engineered IgG binding protein A fragment known as protein Z^{23,24} as a fusion to the p3 protein.²⁵ Second, in this work the circular, single-stranded genome contained within the phage serves as a template for an RCA reaction. Unlike PCR, which has been used to detect phage before,²⁵ the RCA reaction rapidly and exponentially generates dsDNA in a single isothermal step, rather than multiple cycles. Furthermore, because the reaction generates dsDNA as opposed to ssDNA, the products can be easily detected using low-cost fluorescent nucleic acid stains. In addition, the large sequence of the viral DNA enables multiplexing without the need for patterning or expensive fluorescent nucleic acid or DNA probes (Scheme 1). We demonstrate here that using the entire phage genome as an RCA template not only leads to high antigen detection sensitivities via a single isothermal reaction, but that the template can be applied to multiple simultaneous analyte sensing and typing by modifying multiple genomes with unique restriction enzyme sites (Scheme 1).



Scheme 1. Schematic depicting the use of phage and RCA of the phage genomes for antigen detection and typing

Experimental

Rolling circle amplification of phage genomes from whole phage

All solutions used in the reaction are first placed in eppendorf tubes and irradiated in an aluminum foil lined box

approximately 15cm below an 8 watt 254nm UV lamp for 15mins. Phage particles are diluted to the desired concentration in 5ul of HPLC grade water in irradiated PCR tubes. Degenerate thio-phosphate linked RNA hexamer primers are diluted to 40uM in annealing buffer (60mM Tris-HCl pH7.5, 40mM KCl, 16mM MgCl₂) and 5ul is added to each PCR tube. Tubes are heated to 95C for 1min and then allowed to cool to room temperature by leaving on the lab bench for 25mins. dNTPs and polymerase are combined along with buffer to make a 2X RCA reaction mixture (40mM Tris-HCl pH7.5, 80mM KCl, 9mM MgCl₂, 20mM (NH₄)₂SO₄, 8mM DTT, 1mM each dNTP (New England Biolabs), 5U(5ng)/ul phi29 polymerase (Replphi Epicentre)) before adding 10ul to each PCR tube, vortexing briefly, and incubating tubes at 30C for 2hrs. Reactions stopped by heating tubes to 65C for 10mins to denature polymerase.

RCA products were quantified by diluting the 20ul reaction volume to 200ul with TAE buffer (50mM Tris-acetate pH 8.3, 1mM EDTA) and adding SYBR green 1 dye (Lonza) to the manufacturers recommended concentration (1x) immediately before measuring the fluorescence. RCA products could be diluted in TAE buffer and then stored in the fridge for several days before diluting a fraction of the products further with TAE and SYBR green 1 dye. Fluorescence was measured in a QM-6 spectrofluorimeter (Photon Technology International) by exciting at 490nm with a 14watt lamp and recording from 505nm to 550nm with a integration time of 0.5 seconds and photomultiplier set to 1000V. Slit sizes on the monochromators were set to 0.5mm. Alternatively, the products could be digested by adding 20units of PstI-HF endonuclease (New England Biosciences) and incubating at 37C for 1hr, then separating on an agarose gel. PstI-HF reaction buffer (CutSmart Buffer, New England Biolabs) was added to 1x but was not found to be necessary for successful digestion

PstI digestion site mutagenesis on Fzz8 phage

PCR mutagenesis was used to insert an additional PstI recognition site (5'-CTGCAG-3') as a silent mutation into the TetA gene. Briefly, two complementary primers encoding a one bp change in sequence were used to PCR amplify the Fzz8 phage genome using Phusion high fidelity polymerase (New England Biolabs). The PCR products were digested with DpnI endonuclease (New England Biolabs) and was transformed into chemically competent Dh5Alpha cells (New England Biolabs). Resulting transformants were grown and miniprep and the harvested phage genomes were digested with PstI to screen for the proper mutation and sequenced. A similar protocol was used for the insertion of a second PstI site on the M13KE based phage used for phage display. Here the second PstI site was inserted as a silent mutation at the N-terminus of the p8 coat protein.

Anti-Goat Rabbit IgG detection with Fzz8 phage.

25ul of 10mg/ml streptavidin coated magnetic beads (T1, Life Technologies) were diluted in 1ml of PBS with 0.05% Tween-20 (Sigma). The beads were washed three times by magnetically separating the beads and replacing the buffer

three times. Then the beads are resuspended in 250ul of the same buffer and 25ul is aliquoted to each 200ul PCR tube. 1ul of the biotinylated antigen solution diluted in PBS with 1mg/ml Bovine serum albumin (BSA), is added to each aliquot. Tubes are vortexed briefly and then left at room temperature for 1hr with end over end mixing. 75ul of 1mg/ml BSA in PBS with 0.05% tween, was added to each tube to block the excess streptavidin sites and the tubes were incubated for another hour. Fzz8 phage was diluted to 1e12 particles/ml in PBS with 0.1%Tween and incubated for 2hrs at RT with end over end mixing. Beads are magnetically separated from blocking solution and 95ul of diluted phage solution is added and incubated at room temperature with end over end mixing for 1hr. Beads are magnetically separated from phage solution and washed with 200ul of PBS with 0.1% tween 4 times and 100ul of UV irradiated PBS 1 once. 5ul of UV irradiated water is added to each tube to re-suspend the beads and the RCA reaction is performed using the same protocol as phage alone (detailed above).

Phage display

Phage display was performed similar to what has been described previously.²⁶ A library containing 1e9 different phage clones expressing random 12mer peptide sequences on their p3 coat proteins was purchased from New England Biolabs and 1.2e11 phage were incubated for 1hr with 16pMoles of Biotinylated polyclonal anti-lysozyme antibody produced in rabbit (Rockland Immunochemicals) in TBS. The complexes were then immobilized on magnetic streptavidin coated beads (Life Technologies) and unbound phage was removed by washing the beads 10 times with TBS containing 0.1%PBST. Bound phage were eluted using Glycine-HCl solution buffered to pH2.2 and amplified in ER2738 cells (New England Biolabs) following the suppliers instructions. The panning procedure was repeated twice more while increasing the tween concentration in the wash buffer to 0.3% and 0.5%. Individual phage clones were isolated and sequenced following the third round of panning and phage expressing unique peptides were tested for affinity to the antigen.

Peptide cloning to p3 phage coat protein

Cloning specific peptides to the p3 phage coat protein was accomplished via traditional restriction ligation cloning into the EagI and KpnI that are present at the N-terminus of the mature p3 sequence in M13KE type phage from New England Biolabs. The FLAG and c-Myc peptide epitopes were chosen for their reported ability to bind specific antibodies with high affinity.^{27,28} Two complementary oligomers were ordered from Integrated DNA technologies and designed to insert the epitope sequence DYKDDDDK at the N terminus of p3, preceded by the two amino acids DV and connected to the rest of the p3 protein with a tri-glycine linker. The N-terminal DV sequence is the same as in wild-type p3 and was included because previous reports of phage display against the M2 antibody have produced inserts containing the FLAG epitope nested two or three amino acids in from the N-terminus.^{25,26} The c-Myc epitope sequence EQKLISEEDLN was similarly cloned with a tri-glycine linker without further modification. Biotinylated Anti-c-Myc antibody 9E10 was purchased from

Sigma Aldrich. Anti-FLAG antibody M2 was also purchased from Sigma Aldrich, but was biotinylated at a 40:1 molar ratio with NHS-biotin (Pierce Biotechnology) and purified with a 2kDa MWCO mini dialysis cassette (Pierce) before use.

Results

For our initial experiments, we used an Fd-Tet type bacteriophage (carrying tetracycline resistance) engineered to express a repeated IgG binding Z domain of protein A from *Staphylococcus aureus* fused to its p3 proteins (Fzz8). As a first step, the minimal amount of phage needed to produce detectable amounts of DNA within a few hours was determined. For this, we first heated the phage in the presence of degenerate hexamer primers at 95°C, then added a solution of dNTPs and Phi29 polymerase and incubated the samples at 30°C before stopping the reaction by heating to 65°C for 10 minutes.^{27,28,31} Though previous work with phage used 10 minute denaturing times to release the phage genome, we found that heating at 95°C for a minute was not only optimal but that longer heating caused decreases in the subsequent amplification process.²⁵ In order to remove any background DNA contaminants not part of the phage genome, PCR grade clean tubes and tips were used to handle the amplification solutions. In addition, all buffer solutions were irradiated for 15 minutes under a UV lamp before being added to the primer, polymerase and dNTP solutions. In order to confirm that the amplified DNA correlated to the Fzz8 phage genome, the products were reacted with PstI endonuclease, which cuts at a single site within the viral sequence. Using this protocol, phage concentrations from 339 pM to 33.9 aM could be amplified in three hours and digested to produce a distinct band on an agarose gel as shown in Figure 1a. This band migrated at a rate consistent with the predicted ~9500bp dsDNA fragment produced by PstI cutting one site in each phage genome. No other bands were observed in the gel indicating that only the Fzz8 genome was amplified. To better quantify the amplification products, solutions were diluted in TAE buffer containing SYBR green I dye and their fluorescence was measured. The fluorescence peak intensity increased with higher amounts of initial phage used and also correlated with the band intensities seen by agarose gel electrophoresis (Figure 1b).

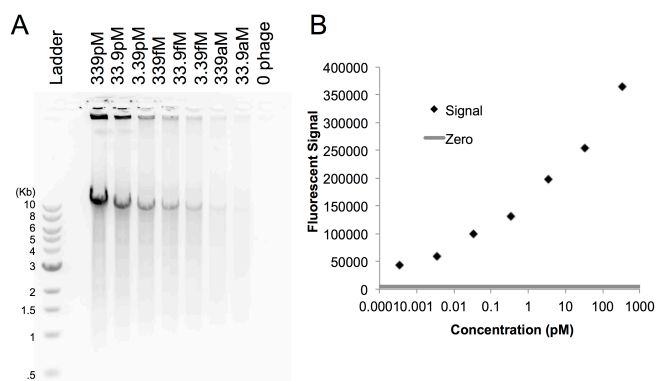


Figure 1. A) Rolling circle amplification products from the indicated phage concentration then digested with PstI endonuclease and run on an ethidium bromide stained agarose gel along with a DNA ladder. Largest DNA ladder band is 10 kilobases. B) Products quantified by fluorescence after mixing with SYBR green I dye.

In order to see if the viral genome could be used to type different antigens in solution, we decided to run RCA reactions from phage that contained genomes that differed from each other in terms of the number and locations of the PstI cut sites. For this, we first inserted a second PstI site in the Fzz8 DNA to generate two types of phage which either contained one or two PstI cut sites. We then ran RCA reactions from mixtures of these two types, followed by a PstI digest and agarose gel electrophoresis. As shown in Figure 2, the relative phage concentration of each type corresponded to the intensity of the bands digested from the amplification products. This was also corroborated by ImageJ analysis of the fluorescent intensities of the bands (Figure S1). Though sequence bias has been shown to occur with RCA due to differences in secondary structure of the template and products,^{32,33} the two 9500bp long phage genomes only differ in a single base, so sequence based amplification bias is highly unlikely to occur. It should however be noted that there was a significant amount of fluorescence visible in the wells loaded with each digest. This was seen in all amplifications and we hypothesize that this is caused by either parts of hyperbranched products not becoming linearized with PstI, or by the highly processive Phi29 polymerase still binding to some part of the products. It is unlikely that this is simply caused by undigested concatemers since extending the digest time or increasing the endonuclease concentration did not decrease the intensity of fluorescence in the well.

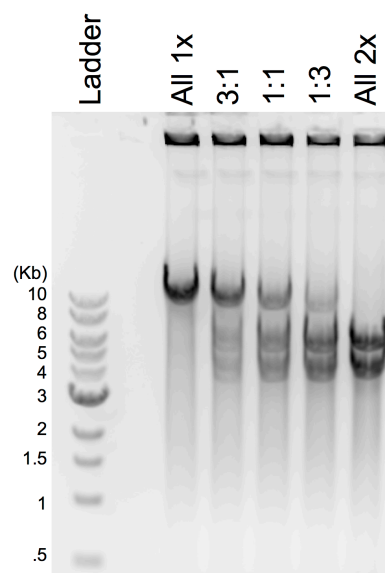


Figure 2. Whole phage particles bearing 1 or 2 PstI recognition sites (1x or 2x) within their genome were mixed at the indicated ratio such that the total phage particle concentration was 339pM and then amplified with RCA. Amplification products were digested with PstI endonuclease and run on an ethidium bromide stained agarose gel.

To demonstrate the utility of phage as biosensor probes, we first tested the use of Fzz8 for detecting rabbit IgG1 through binding of the repeated Z domain to the Fc portion of the antibody. The Z domain is known to bind rabbit IgG1 with a dissociation constant slightly higher than the nM range with which it binds human IgG1.^{34,35} In the assay, varying concentrations of biotinylated rabbit IgG1 were first bound to streptavidin functionalized magnetic beads in PCR tubes and then probed with a solution of 1.67nM Fzz8 phage whose genome contained a single PstI cut site. Non-specifically bound phage were removed by washing several times with a 0.1% tween solution. The RCA reactions were next run directly from the phage coated beads by first adding the primers directly to the same PCR tubes and heating at 95°C followed by the polymerase, dNTPs and heating at 30°C for 2 hours. The RCA products were then mixed as before with SYBR green and the fluorescence output of each tube was measured. As shown in Figure 3, the RCA reactions allowed us to detect down to ~3pM of rabbit IgG in solution demonstrating the high sensitivity of the assay. The RCA products were also digested with PstI and characterized by gel electrophoresis to demonstrate that the phage genome was the amplified product.

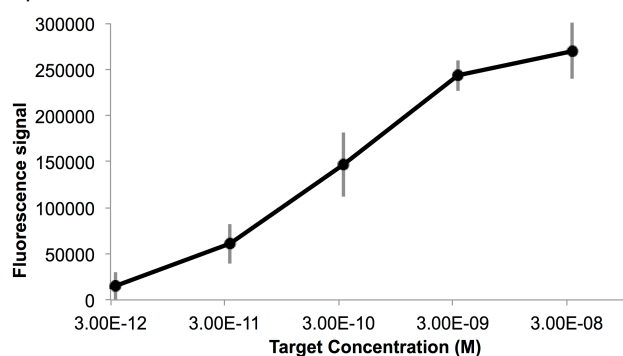


Figure 3. Results of an assay for Rabbit IgG using phage expressing the antibody binding ZZ domain as RCA probes. Signals represent averages and standard deviations of four assays normalized to the no-target control in each assay. Amplification products are quantified by mixing with SYBR green 1 dye.

In order to compare the RCA results with phage ELISA data, we first reacted Fzz8 phage with beads coated with different concentrations of rabbit IgG. Next, we added horseradish peroxidase (HRP)-conjugated anti-Fzz8 (anti-M13) antibodies (GE Healthcare) and developed a signal with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). As shown in Figure S2, the phage ELISAs gave comparable detection limits to those observed with the RCA reactions. Phage ELISA is known to be very sensitive because each virus can bind multiple anti-Fzz8 (anti-M13) antibodies, each of which in turn has multiple HRP enzymes. However, in contrast to phage ELISA, using RCA does not require the use of additional detection antibodies. Furthermore, because each phage genome can be typed to a specific analyte, different antigens can be identified in a single solution, which is also not possible with ELISA.

Next, in order to use phage RCA to detect and identify multiple proteins simultaneously, we created three types of

phage, each capable of binding different proteins with high specificity. Each set of phage was designed to contain different patterns of PstI recognition sites within the genome. For multiplexed sensing, we first produced phage that recognized anti-FLAG, anti-cMyc and anti-lysozyme antibodies. For the anti-FLAG and anti-cMyc targets, we cloned specific peptides that are known to bind the two different antibodies as fusions to the p3 proteins.^{36,37} The phage that recognized anti-lysozyme antibodies were isolated by phage display. Sequencing of ten plaques eluted from a fourth round of panning against anti-lysozyme gave the consensus sequence TDFNTMAKNNPP. All three types of phage were next tested in ELISAs for binding to anti-lysozyme, anti-FLAG, and anti-cMyc, and in all cases, each phage type was found to bind only their specific targets with minimal cross-reactivity. To gauge the binding affinity of each phage against their specific targets, ELISAs were performed with serial dilutions of each target. Based on these results (Figure 4), the anti-FLAG binding phage showed the highest affinity while anti-lysozyme binding phage showed the lowest.

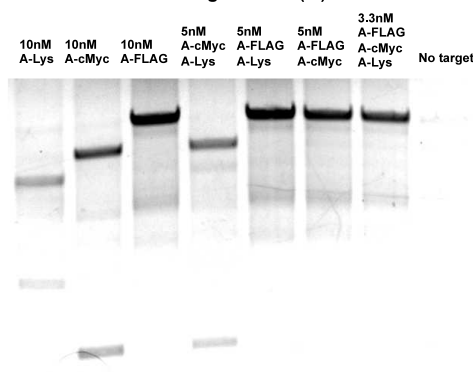
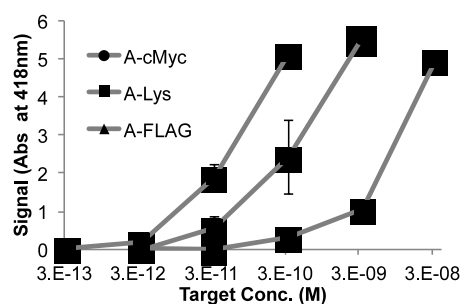


Figure 4. (Top) Comparison of three ELISAs using peptide bearing phage at a concentration of 1.66nM to probe varying concentrations of their respective targets. Results are listed by target. Bound phage were detected with an Anti-M13 HRP conjugate. (Bottom) Gel electrophoresis results using equal molar concentrations (~550pM) of each phage type.

In order to differentiate the phage genomes, PCR mutagenesis was used to modify the DNA of the anti-lys and anti-cMyc binding phage by making silent mutations at different points. A second PstI site was made in the p8 and p4 coding sequences for anti-lys and anti-cMyc binding phage, respectively. This was designed so that after the PstI digest, the anti-lys binding phage would produce two fragments composed of 4876 and

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2345 bases while the anti-cMyc binding phage would produce two fragments composed of 5847 and 1375 bases. The anti-FLAG binding phage was designed to contain a single PstI site. Successful mutations were confirmed by digest and gel electrophoresis, and all mutated genomes produced phage with no noticeable loss in efficiency. Next, ~550 pM solutions of the three different sets of phage were used to detect varying amounts of the three biotinylated targets mixed in a single solution. For this, we first reacted the streptavidin-coated beads with 10 nM solutions that contain a single antigen or a mixture of the three different proteins. After the unbound phage were removed by washing, RCA reactions were run for 2 h and the products were measured by photoluminescence and agarose gel electrophoresis. When starting from equimolar amounts of the three different sets of phage in solution, the RCA products gave the same fluorescence output. However, the gel electrophoresis results revealed that the varying phage affinities for their specific antibody targets greatly affected the detection limits for each antigen in that the phage that had the highest affinity for the antigen tended to dominate the gel results. For example, since the anti-FLAG binding phage demonstrated much higher binding affinity than the anti-lys binding phage, the RCA product from the anti-FLAG binding phage was overwhelmingly brighter than that obtained from the anti-lys binding phage gel electrophoresis despite the equal loadings of antigens in solution. Thus, the brightness of each band did not correlate with the actual antigen concentrations (Figure 4).

Because of difficulties in creating phage with equal affinities for each target, we decided to tune the starting concentrations of each phage type in the assay to match their specific binding affinities. Since the anti-FLAG binding phage clearly showed the highest affinity by ELISA (Figure 4), we lowered the starting anti-FLAG phage concentration to 1.66 pM. The anti-cMyc binding phage showed ~100 fold lower affinity than the anti-FLAG phage and ~10 fold higher affinity than the anti-lysozyme binding phage so their concentrations were changed to 166 pM and 1.66 nM, respectively. The average concentration of the entire phage mixture was thus ~610 pM, similar to the concentration used in the previously described assay (Figure 4). Next, the phage were mixed at varying dilutions with the beads coated with different antigens, followed by RCA, photoluminescence measurements (Figure S3) and gel electrophoresis (Figure 5). By matching the starting phage concentration to their relative binding affinities for the targets, we were able to see that DNA band intensities obtained now correlated to the starting concentrations of antigens in solution (Figure 5). This relationship was also confirmed by ImageJ analysis. It is also important to note that the assays were highly specific to the particular antigens tested, meaning no extraneous bands were observed in the absence of a specific target.

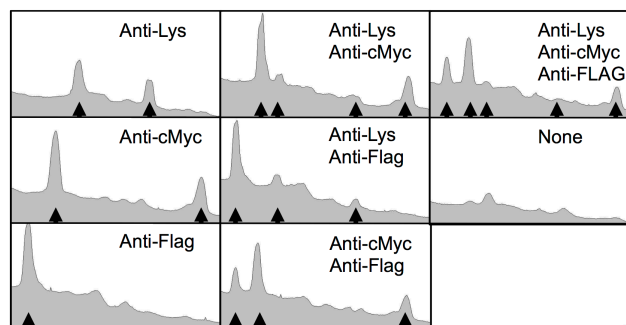
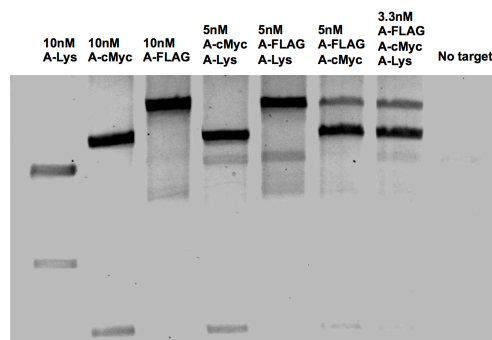


Figure 5. (Top) Gel electrophoresis results of using an unequal mixture of the three phage sets to detect mixtures of targets at the indicated concentrations. The phage concentrations used were based on the relative affinities determined by phage ELISAs. The concentrations of the anti-lysozyme, anti-cMyc and anti-FLAG binding phage were at 1.66nM, 166pM and 1.66pM respectively. (Bottom) imageJ analysis of gel shown. Peaks corresponding to bands created from phage products are indicated with arrows.

Conclusions

In conclusion, we have demonstrated the use of an engineered bacteriophage as probes to detect multiple antigens in solution using the contained viral genome as a detectable agent. The large genome of the virus was easily amplified via RCA, and could be engineered to not only express specific binding domains (peptides, antibodies) to particular antigens, but also contain restriction enzyme sites for antigen typing by gel electrophoresis. This method allowed sensing for different antigens in solution without expensive fluorescent nucleic acid probes or a patterned surface. We show that the isothermal phage RCA reactions yield sensitivities similar to those obtained via phage ELISAs, but without antibody or enzyme conjugates. Finally, we demonstrate that despite different affinities for protein targets, the initial phage concentrations could be altered to obtain quantification of multiple analytes in solution. In future work, we will study ways to minimize the differences in phage binding affinity by using phage-antibodies using non-canonical amino acids for covalent photo-conjugation of antibodies to the ZZ domain expressed on Fzz8.^{38,39}

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