

**APTAMERS AND PHAGE AS TOOLS FOR THE DEVELOPMENT
OF NOVEL AND SENSITIVE POINT-OF-CARE DIAGNOSTICS**

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By
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APTAMERS AND PHAGE AS TOOLS FOR THE DEVELOPMENT OF NOVEL AND SENSITIVE POINT-OF-CARE DIAGNOSTICS

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ABSTRACT

Immunochromatographic lateral flow assays (LFAs) represent a well-established point-of-care diagnostic analytical method for the primary testing of diverse samples. The sensitivity of conventional LFAs that use the standard colored nanoparticles as reporters lags behind the more elaborate laboratory techniques, such as plaque counting, PCR or ELISA. To address this issue, we have introduced an innovative approach that utilizes functionalized M13 bacteriophage nanoparticles as reporters in the lateral flow diagnostic system. M13 phage offers a multitude of binding sites on its major coat protein pVIII for reporter enzymes, and for affinity agents specific for the target of interest. Furthermore we employed SAM-AviTag phage, derivatives of phage M13 in which the N-terminus of the tail protein pIII is replaced by the enzymatically-biotinylatable AviTag peptide (GLNDIFEAQKIEWHE). The lysine residue (K) in the AviTag is a substrate for biotinylation by the *E. coli* biotin ligase (BirA) enzyme. Using streptavidin or Neutravidin, any biotinylated affinity agent can then easily be linked to these enzymatically-biotinylated phage particles. Viral nanoparticles were functionalized with target-specific antibodies and multiple copies of an enzymatic reporter (horseradish peroxidase). These particles were successfully integrated into a lateral-flow immunochromatographic assay detecting MS2 virus, a model for viral pathogens. The limit of detection of the assay was 10^4 MS2/mL, 1000-fold more sensitive than the conventional gold nanoparticle

lateral flow assays, and results could easily be evaluated, even without advanced lab instruments.

Aptamers are short, library-selected nucleic acid molecules that can recognize and bind to pre-selected targets with high affinity and selectivity. They have been used as recognition elements in a variety of applications. We screened for DNA aptamers specific to the murine anti-lysozyme antibody, HyHEL-5. During the validation process, however, the originally selected aptamers did not show successful binding. As an alternative, literature DNA aptamers were employed to construct phage displaying aptamers and HRP, which were used as LFA reporters. We describe the first modification of bacteriophage particles with aptamers as bio-recognition elements, and demonstrate their use in ultrasensitive lateral flow assays detecting IgE and PBP2a (Penicillin binding protein) of *Staphylococcus aureus*.

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Chapter 1: **INTRODUCTION**

1.1 Theoretical Background

In recent years, advancement in diagnostic technologies has led to new platforms for the analysis of biological agents. For example, several diagnostic tools have been developed for the detection of biological threat agents that are based on genetic (e.g., Polymerase Chain Reaction (PCR), DNA sequencing, hybridizations and microarray) and immunosensing techniques (1,2). Immunoassays constitute one of the largest classes of diagnostics systems, and rely on the inherent ability of an antibody to recognize and bind a specific antigenic epitope. ELISAs have been the gold standard for detecting protein molecules and other biological targets (e.g., toxins, viruses) for the past 40 years and have been a valuable tool in biochemical research and diagnostics (3). In general, an immunoassay commonly consists of capture antibodies that are immobilized on a solid support (such as a microtiter plate or a magnetic bead), which captures the target analyte. The surface-captured analyte is then detected by binding an antibody conjugated to an enzyme reporter. An ELISA, like all other immunoassays, depends on molecular recognition, where a molecule such as an antibody or a DNA probe, binds or hybridizes to its target. The other key component of the assay is the transduction of this molecular recognition event into a readable output signal. Enzymatic reporters are most commonly used for generating the output signal in the form of absorbance, fluorescence, chemiluminescence or bioluminescence from appropriate substrates and detected with the proper instrumentation. However ELISAs are generally confined to a small

dynamic range, they use elaborate instrumentation, and the sensitivity with which the enzymatic label can be detected constrains its sensitivity (4).

Developments in DNA amplification techniques (e.g., PCR, RT-PCR) have revolutionized diagnosis and research when nucleic acids are the target, but still there are limited techniques for equally sensitive detection of non-nucleic acid biomolecules. The introduction of immuno-PCR methods is an appealing concept to bridge the gap and has been exploited repeatedly since its original development (5). Immuno-PCR potentially combines the advantages of both ELISA and PCR. It employs a chimeric molecule consisting of an antibody (the primary recognition element) conjugated to an amplifiable DNA reporter. So amplification of the reporter DNA serves as the transduction element of the molecular recognition. Immuno-PCRs have shown to achieve 100-10,000-fold increased sensitivity compared to standard ELISAs, but the method still has failed to establish itself in the analytical laboratory, as it suffers from several drawbacks such as complicated preparation of immuno-PCR reagents, non-specific binding of DNA, and lack of reproducibility (6-8).

Despite multiple innovations that have boosted assay sensitivity to new levels, a typical immunoassay assay still requires large laboratories, trained technicians, multiple steps and the use of expensive instruments, e.g., a spectrophotometer for signal detection. This limits its use to hospital or outsourced laboratories and often leads to days of waiting for lab test results. Therefore there has been enormous demand for developing very rapid, sensitive, and inexpensive

analytical tools. To achieve this goal, there have been increasing efforts toward bringing infection diagnostics closer to the patient for rapid identification of agents at the point-of-care (POC).

Point-of-care detection allows diagnoses in the hospital, at home, or in the physician's office and in addition gives immediate results to better support patient care. It emphasizes early detection of disease and better management of chronic conditions. Among the many possible POC methods, the immunochromatographic lateral flow assay (LFA) has gained enormous attention due to its simplicity and versatility. The technology employs a test strip that collects sample through lateral flow, and detects the presence of a target molecule through a specific antibody that is labeled with a colorimetric indicator. LFAs are a major class of rapid diagnostics and have dominated the POC market over the last three decades and is a multi-billion dollar market at present (9). The first popular application of a LFA was the pregnancy test for the detection of human chorionic gonadotropin (HCG) in pregnancy (9). LFAs are well established, and provide inexpensive analytical methods for the primary screening of various samples. They provide a quick qualitative or semi-quantitative result without the need for elaborate sample preparation or capital investment. In conventional LFAs colloidal gold, selenium, blue latex or carbon-nanoparticles-bearing antibodies to the target are used as the reporter, which is analyte-bridged to a capture antibody immobilized onto the surface of a nitrocellulose strip. The visual confirmation of a line of captured reporter particles indicates a positive result, whilst the appearance of the control

line further downstream demonstrates that the liquid has flowed correctly (Figure 1-1 depicts the scheme of a typical lateral flow assay).

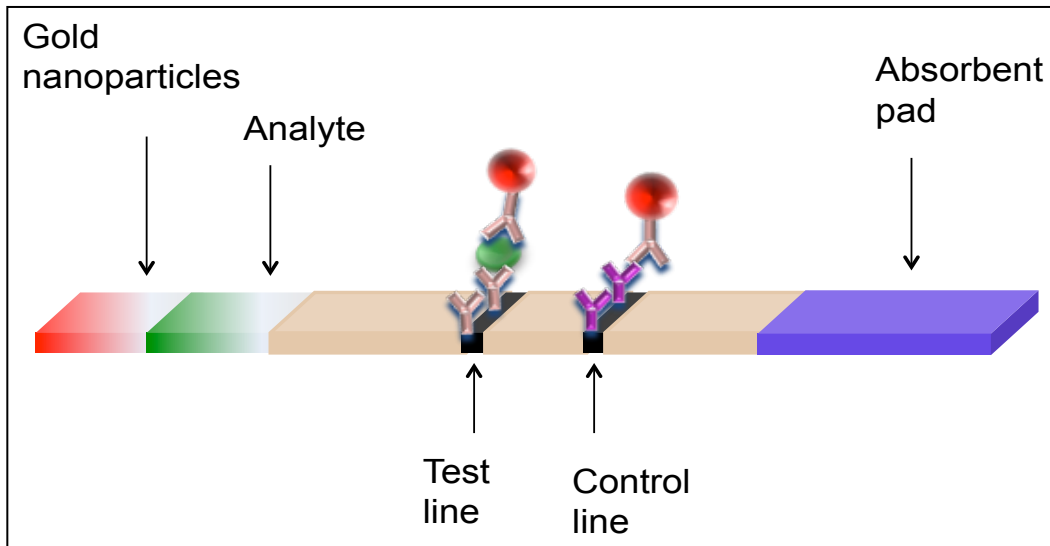


Figure 1-1: Scheme of lateral flow assay

LFAs have been used to detect a wide range of biomolecules, including nucleic acids, proteins, toxins, and hormones, as well as bacterial and viral pathogens (10-12). The classic LFA is a rapid and easy-to-use immunoassay. Nevertheless, the sensitivity of LFA lags behind more elaborate laboratory methods used for proteins (e.g., ELISA) and viruses (e.g., plaque assays or PCR) (13-15). To illustrate typical sensitivities (which this work has greatly improved upon), colloidal gold LFAs for Japanese encephalitis virus in swine are reported to detect 2.5×10^6 pfu/ml and a comparable assay for filamentous *Escherichia coli* M13 phage was able to detect 5×10^7 pfu/ml (16,17).

Bacteriophage, viruses that infect bacteria, are now playing a role in diverse applications including diagnostics (18). Functionalized viral nanoparticles have been shown to be suitable affinity reagents in immunoassays (19-21). They present versatile scaffolds to accommodate diverse recognition (aptamers, antibodies, lectins, etc.) and reporter (enzymes) elements leading to universal, ultrasensitive bio-detection reporters. In this dissertation, we present a highly sensitive and specific detection assay using functionalized viral nanoparticles as reporters in LFAs for viruses and proteins. The present work overcomes the sensitivity limitations that currently limit LFA performance compared to lab-based immunoassays (22). M13 bacteriophage is introduced as scaffold for modification with anti-analyte antibodies and enzyme reporters. Two different approaches are employed to manufacture antibody and peroxidase-doubly-modified M13 bacteriophage. In the first, horseradish peroxidase (HRP) and target specific antibodies are covalently attached to the coat protein (pVIII) of the *E. coli* bacteriophage M13. In the second approach, SAM-AviTag phage, derivatives of phage M13, where the N-terminus of the phage coat protein pIII displays the enzymatically-biotinylatable AviTag peptide (GLNDIFEAQKIEWHE), are employed. The lysine residue in the AviTag is a substrate for biotinylation by *E. coli* biotin ligase (*birA*). Using streptavidin or neutravidin, any biotinylated affinity agent can then easily be linked to these enzymatically-biotinylated phage particles (23-25). Both approaches are suitable for the attachment of intact antibodies (rather than the single-chain variable fragments (scFvs), which require cloning and often have lower affinity than the parent

antibody). The use of bacteriophage as a scaffold for multiple enzyme reporter attachment in lateral flow assays comprises a novel and innovative approach and shows a superior sensitivity over conventional lateral flow assays and we anticipate that it can have an immediate impact in the areas of point-of-care detection.

1.2 Introduction to Lateral Flow Assays (LFAs)

LFAs are a well-established, inexpensive point-of-care diagnostic analytical method with proven utility for the primary testing of diverse samples. This mode of detection has gained a lot of attention in recent years due to its ease of use, rapid time to result, and minimal laboratory equipment requirements (17). The scheme in lateral flow technology (LFT) is similar to ELISA, but the base substrate is a nitrocellulose membrane bearing a capture reagent, usually an antibody or antigen. The presence of target molecule is detected by a second specific antibody labeled with a colorimetric indicator. The most common labels employed in LFAs are colored nanoparticles such as selenium, blue latex, colloidal gold, carbon-nanoparticles, and recently upconverting phosphors (26-28). LFAs are commercially available for various targets including infectious agents such as bacteria, viruses, hormones, and drugs (28-35). The utilization of the membrane strip as the immunosorbent and directly observing the results by naked eye serves as an analytical platform that provides one-step, rapid and low-cost analysis.

1.2.1 Basic principle and assay formats

LFA strips are designed to detect the presence of an analyte that can be any antigen or antibody by a specifically labeled antigen or a labeled antibody. The LFA test strip consists of a sample pad, a conjugate pad, membrane and an absorbent pad that are laminated into a sheet of plastic backing. The sample pad holds the sample fluid and allows diffusion of sample into the conjugate pad that is infused with labeled detector reagent. If the sample contains the analyte, it will bind to the detector reagent and the complex will flow and bind to the capture reagent (antigen or antibody) at the test line on the membrane. The binding of the analyte to the detector gives a colored line indicating a positive result. Appearance of colored line at the control line ensures the proper functioning of the test. The absorbent pad at the end of the strip wicks the fluid through the membrane assuring a continuous flow and thus maintains a clear background.

LFAs are available in different possible formats depending on the type of target analyte. The most-commonly used formats are the competitive and the sandwich assay. In the competitive assay format, the reporter binds directly to the immobilized ligands. Depending on the type of competitive assay used, the target analytes may bind either to the reporter particles or to the immobilized ligands. In both cases, the presence of the target impedes the binding of the reporter to the capture antibody on the test line. Therefore, in the absence of target in the sample fluid a signal will be observed on the test line. If the target is present, it will bind to

the reporter that in turn would not be able to bind on the capture antibody resulting in a weak or no signal and indicating the presence of target analyte in the sample (Figure 1-2a). Competitive assay format are used often when test analytes have a low molecular weight or have a single antigenic determinant (36). In a sandwich assay two different antibodies (polyclonal or monoclonal) are used; both of which can bind to the analyte at distinct epitopes. The first antibody is immobilized on the surface at the test line where it captures the analyte. The second antibody that is labeled serves as the detector reagent, after incubation with the sample analyte binds to the analyte forming a sandwich with the analyte and the first antibody (Figure 1-2b). Sandwich assays are useful for analytes that present several antigenic epitopes, for example viral proteins (37).

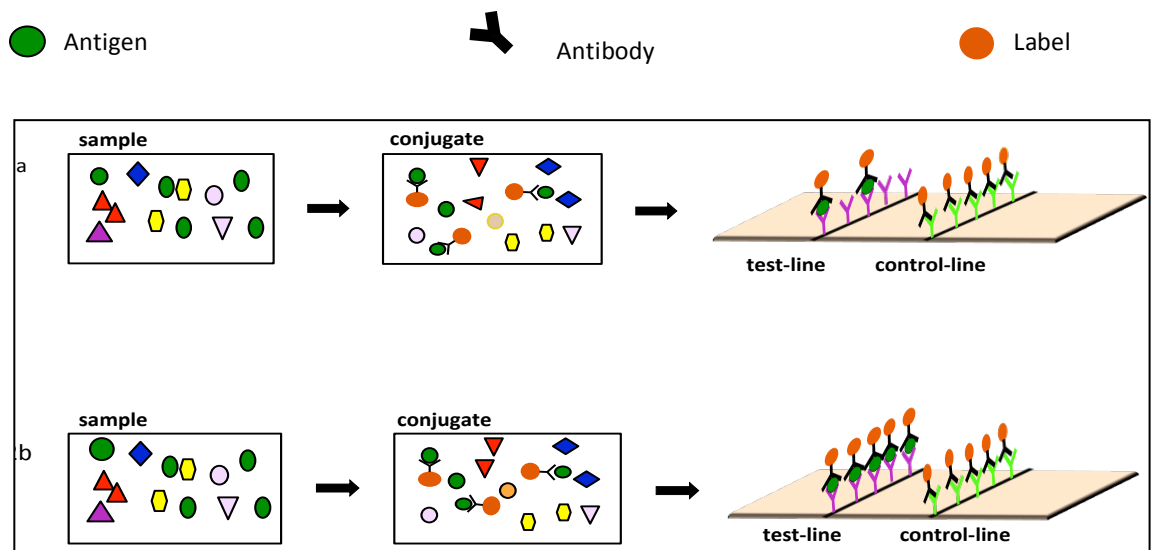


Figure 1-2: Common immunoassay formats in lateral flow assay

a) Competitive immunoassay, b) Sandwich immunoassay.

1.2.2 Membranes in LFA

The membrane is a critical component of the LFA. Nitrocellulose has been the material of choice in most LFAs systems. Several other materials have also been introduced including polyether sulfone, cellulose acetate, nylon and polyvinylidene fluoride (PVDF) membranes. However these materials could not establish their place due to several factors including cost, limited utility, lack of understanding the new chemistry and processing requirements, and also due reluctance in change due to existing experience in the use of nitrocellulose.

Nitrocellulose: The history of nitrocellulose as a binding membrane started in the 1960 and since then nitrocellulose has been widely used in many applications from blotting to protein arrays and immunoassays (38). While considering nitrocellulose in LFA, it has certain characteristics that make it useful and the only successfully and widely applied material to date. These characteristics include its low cost, good capillary flow characteristics, high protein-binding capacity and a variety of available products with varying wicking rates, pore size and surfactant contents. The membrane serves as a critical function in LFAs including stable binding surface to capture antibodies on the test and control lines, controlling the diffusive and capillary flow of the mobile phase. Due to the inherent hydrophobic nature of nitrocellulose, it has a high adsorptive capacity for proteins. The proteins are usually attached to the membrane by drying at room temperature. The binding

forces that bind them on the membrane arises from hydrophobic interaction and electrostatic interactions (39) and studies have suggested that the binding is through non-covalent, hydrophobic interactions (40). However in order to function as the reaction matrix in a lateral flow system, the membrane must be hydrophilic and have consistent flow characteristics. Therefore, this hydrophobic nature of nitrocellulose is not desirable when reporters functionalized with antibody molecules are flowing through it, since these antibodies can attach to the membrane non-specifically. This non-specific binding can be prevented by blocking with a variety of reagents (e.g., bovine serum albumin (BSA), surfactants such as polyvinylpyrrolidone (PVP), Tween or Triton). Some membranes are also manufactured with added detergents to ensure even wetting. Nitrocellulose also possesses some properties that make it undesirable for LFAs which include non-reproducibility of performance, shelf-life issues, flammability (primarily in unbacked membranes), sensitivity to humidity and being subject to breakage due to its brittleness and compression.

Fusion 5 as an alternate material for LFA: There is a need for novel materials to improve the functional performance of lateral flow assays. Materials that can perform multiple functions, i.e., can act as sample application area, reaction surface, separation medium, and wick all in one. This could be very beneficial in terms of simplicity and cost cutting. In this aspect Fusion 5, a new material from GE healthcare, made of glass fiber and intended to serve in multiple roles can be used as

a replacement for all the solid materials used in a LFA. It allows entire lateral flow test to be built upon a single material, simplifying process and providing a cost saving to manufacturers. Fusion 5 is a hydrophilic glass fiber membrane with a permanent negative charge. Proteins can be adsorbed onto Fusion 5 when spotted at $\text{pH} < \text{pI}$ (e.g., in acetate buffer at pH 3.6) and successive drying can make them stick permanently (41). It overcomes some of the limitations observed with traditional nitrocellulose that can affect the wicking rate and protein-binding characteristics of the membrane with time, which in turn can have a significant effect upon long-term assay performance. Also the hydrophobic nature of the nitrocellulose membrane can have adverse effects on the test results if it binds to other proteins non-specifically. Even though certain surfactants or polymers can overcome this issue, the presence of these surfactants and polymers can interfere with the test assays, particularly when the immunogenic interactions are needed for proper functioning of the assay since, the high levels of hydrophilic blocking materials can result in false positive or negative results. Fusion 5 has very fast wicking rate, so entire assay times can be reduced, and it also has a low background issues. Being hydrophilic in nature, it does not require extensive blocking or careful storage conditions and apart from that, it does not substantially age on storage. Fusion 5 is a novel approach to substrate design that can represent a possible alternative specifically for application in point-of-care assays.

1.2.3 Commonly used reporters in LFAs

Reporters are the key components of the LFA system since they allow visual detection of antigens or antibodies. With the advancement in detection technology and instrumentation, the development of labels or markers has also grown. Colloidal gold is one of the most commonly used reporters in lateral flow assays (11). It is easy to prepare in the laboratory and gives intense color and does not require any development process for visualization. It is very stable and does not bleach after staining on membranes. Colloidal gold is readily available from commercial sources in unconjugated and conjugated forms.

Other reporters such as colored latex, selenium (42), or paramagnetic particles are also used. In recent years, fluorescent and luminescent labels have been used in various assays that have resulted in very sensitive detection and now these labels have been incorporated successively into lateral flow formats (43,44). Latex particles are one of the earliest and most versatile reporters used in LFAs that are easily used for conjugation, including simple adsorption and covalent coupling through amino, carboxyl, and thiol groups. These latex particles can also incorporate color dyes including fluorescent dyes, and paramagnetic media. The use of paramagnetic particles in LFAs is gaining much attention these days. They are colloidal particles of iron oxide and can be coated by polymers that help in the adsorption and covalent linking of antibodies and antigens. Measuring the magnetic flux generated when placed inside a magnetic field does the detection and

quantitation of paramagnetic particles. Enzyme labels attached onto gold nanoparticles (e.g., HRP) have also been reported to successfully enhance the signals from gold nanoparticles in LFAs (45).

In general, reporters used in the LFAs should possess some features that are favorable for the immunoassay, such as their ability to remain stable under varying conditions such as in humid conditions. A relative small size of the labels is preferred over a large size to allow flow through the membrane structure.

1.3 M13 Bacteriophage

M13 (or f1) phages are filamentous phages that belong to the family Inoviridae. They are non-lytic phage that infect *E. coli* through pili and are metabolically inert in their extracellular form. They reproduce only by infecting and exploiting the bacterial host's metabolic machinery. Once infected, the viral genome is inserted into the host cell, where it directs the production of new progeny phage. (46,47). M13 phage are about 900 nm long and 6 nm in diameter. Their genome consists of circular single stranded DNA (ssDNA) which is 6407 nucleotides, enclosed in ~2,700 copies of the major coat protein pVIII. There are other minor coat proteins that are present on the ends of phage: five copies each of pVII and pIX at one end while the other end is capped with 5 copies of pIII and pIV (Figure 1-3). The M13 genome is composed of 11 genes from which 5 genes encode the coat proteins and 6 genes encode the proteins responsible for replication and virus assembly. The phage

coat is the product of a 50 amino acid protein, pVIII, which is encoded by gene VIII (or g8) in the phage genome and is entirely alpha-helical in structure so that the molecule forms a short rod (46). This rod contains three distinct regions: a negatively-charged region at the amino terminal end containing acidic amino acid residues that forms the outer, hydrophilic surface of the virus particle, a basic, positively-charged region at the carboxyl terminal end lining the interior of the protein cylinder and a hydrophobic region for the formation and stabilization of the phage particles.

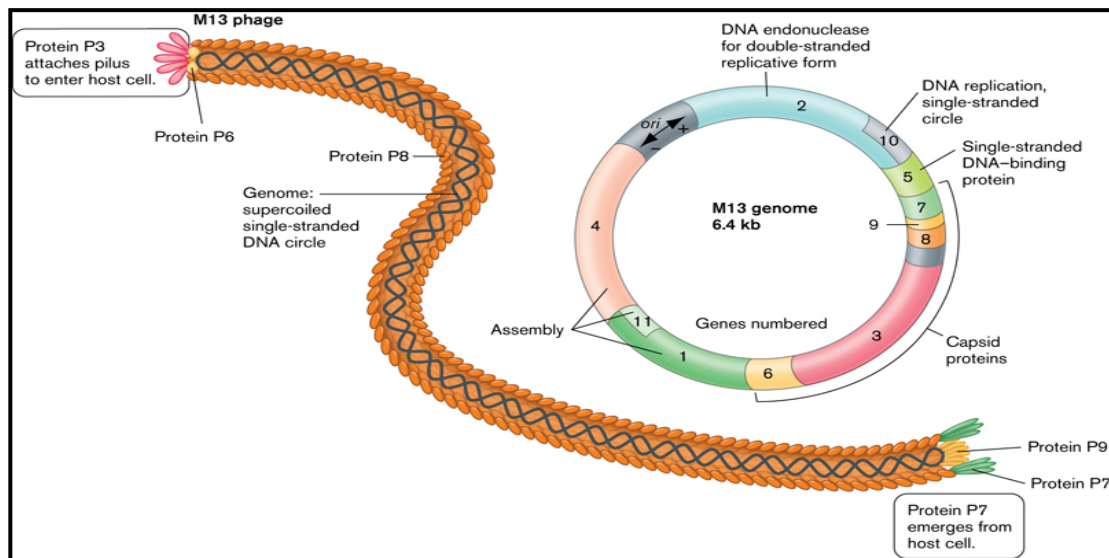


Figure 1-3: The M13 phage structure and genome (47)

M13 life cycle: The infection of *E. coli* begins when pIII attaches to the F pilus of *E. coli*. The pilus is then withdrawn until the phage reaches the surface of bacteria and pIII binds to the *E. coli* integral membrane protein, TolA (48) and the phage genome

is transferred after contacting the bacterial surface. The infecting phage disassembles, and the coat proteins get inside the bacterial membrane (49). The phage genome is converted to double-stranded DNA (dsDNA) by bacterial enzymes and the synthesis of M13's 11 proteins begins. The virion assembly begins with the accumulation of phage proteins and ssDNA. Proteins pVIII, pVII, pIX, pVI, and pIII insert into the inner bacterial membrane upon synthesis. pV (single-strand binding protein) coats the newly synthesized phage ssDNA and prevents their conversion to dsDNA. This DNA-protein complex interacts with the integral membrane proteins (pI and pXI). This in turn results in the simultaneous assembly and secretion of viral particles using a pool of phage coat proteins that have already been inserted into the inner membrane. The viral particle is then released from the cells, without killing them (50).

Applications of M13 phage in phage display: The first reports of filamentous phage in genetic manipulation was shown in 1985 by Smith (51) where he showed that the insertion of a foreign DNA fragment into the phage genome yielded a phage particle displaying the foreign polypeptide sequence as a fusion to the coat protein on its surface and this displayed peptide was specifically recognized by an antibody. The phage displaying the peptide could be enriched from a mixture of wild-type phage and display-phage particles. The displayed molecule had a specific DNA sequence that could be studied by DNA sequencing. These studies by George Smith in 1985 are considered to be the first description of phage-display technology.

Further studies demonstrated that properly folded and functional proteins were also displayed on the surface of filamentous phage successfully (52,53). Phage allow a wide range of molecules to be displayed on their surface and in this light the display of antibody fragments is of particular interest since antibodies have applications in diagnostics, biological chemistry and therapeutics (54-58).

AviTag Phage: Conventional phage display relies on the insertion of foreign DNA fragments in the phage genome and thus displaying peptide sequences that can be recognized specifically by certain antibodies. Phage display requires both the peptide and the DNA fragment to be of a known sequence. This technology may pose a disadvantage if phage display is used in diagnostics because in that case the entire sequence of the antibody has to be known. Even if employing single chain variable fragment (scFvs), it requires cloning and usually have lower affinity than the parent antibody. ScFvs are variable regions of the heavy and light chains joined together by a short peptide linker that represent the complete binding site of an antibody. In phage display these scFvs are made to express the antigen-binding domain as a single peptide.

Studies have shown that phage can be made to express a certain peptide that can be altered enzymatically (59). SAM-AviTag phage are the derivatives of M13 phage containing an AviTag peptide on the phage coat protein pIII (23-25). The AviTag is a 15-amino-acid peptide and was cloned and displayed at the N-terminal end of protein pIII, which resulted in proper insertion into the phage genome

without affecting the efficiency of cloning reproducibility. Furthermore, the lysine (K) residue within the peptide sequence is the substrate for biotinylation with the biotin ligase, BirA, enzyme. Any biotinylated affinity agent then can easily be linked to these enzymatically biotinylated phage particles using streptavidin or neutravidin (25,60). The interaction between avidin and biotin is the strongest among non-covalent interactions with a dissociation constant of 10^{-15} M (61) and can be used in various applications including the detection of target molecules (62).

1.4 MS2 bacteriophage

Bacteriophage MS2 belongs to the family Leviviridae and is a small, non-enveloped icosahedral, male-specific bacteriophage of *E. coli* (Figure 1-4a). The MS2 virus has a 27 nm protein capsid that surrounds a single-stranded RNA genome of 3,569 nucleotides, one of the smallest known genomes. The MS2 genome was the first genome to be sequenced completely (63). It encodes four different proteins: coat protein, a maturation protein (or A protein), a replicase subunit, and a lysis protein. The coat protein of MS2 is its primary structural component. The A protein is responsible for attachment to the bacterial pilus, replication, RNA packing, and infectivity. The replicase and lysis proteins help in replication and the lysis of the *E. coli* bacteria, respectively (64). The MS2 virion consists of one copy of the maturation protein and 180 copies of the coat protein (organized as 90 dimers) that

are arranged into an icosahedral shell protecting the genomic RNA inside. The coat protein is a five-stranded β -sheet with two α -helices and a hairpin (Figure 1-4b).

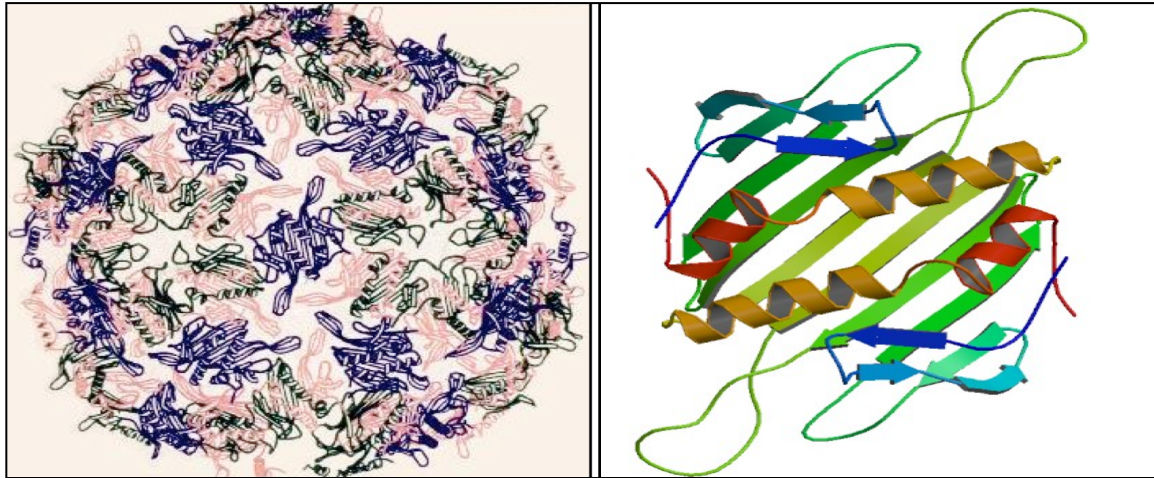


Figure 1-4: (a) Structure of MS2

Source: <http://xray.bmc.uu.se/~karin/ms2.html>

(b) Crystal structure of MS2 coat protein dimer (Source: Protein Data Bank (PDB)).

MS2 has been widely used as a model for viral pathogens and is used as an indicator of water quality by the United States Environmental Protection Agency. They have been used as simulants in place of small RNA virus (e.g., Ebola virus, Marburg virus, and the equine encephalitis alphaviruses), due to their small size, simple structure and harmlessness to humans and animals. It also has been used as a model organism for many molecular processes such as viral replication, translation, infection, and assembly (65,66). MS2 has also been studied for testing and development of systems for the detection of biological warfare agents (64,67-69). Furthermore,

genetically modified forms of MS2 are available that have been studied in clinical diagnostic tool and vaccine development (70-73).

1.5 Aptamers

Nucleic acids are attractive molecules for combinatorial chemistry. They can be folded into well-defined secondary and tertiary structures and can be easily amplified. This property of the nucleic acids has been adopted in selection of high affinity ligands against various targets via the process of Systematic Evolution of Ligands by EXponential enrichment (SELEX). The technology of SELEX was first proposed by Tuerk and Gold in 1990 (74). They used a combinatorial nucleic acids library to select RNA oligonucleotides that were able to bind T4DNA polymerase gp43 very tightly and selectively. Then later Ellington and Szostak (75) used the same technology to isolate RNA and DNA ligands against some targets and named these ligands as “aptamers”. Aptamers are short single-stranded functional nucleic acid binding molecules that are capable of binding to specific targets and distinguish those targets discreetly. The functionality of aptamers is based on their ability to form three-dimensional structures such as stems, loops, bulges, hairpin structures, pseudoknots and G-quadruplex structures.

The SELEX process is an iterative process of *in vitro* selection and enzymatic amplification that drives the selection towards relatively few but high affinity structural ligands (76). The starting point of the SELEX process is a chemically

synthesized random DNA or RNA library. Typically such a library consists of 10^{15} unique sequences comprising a central random region flanked by conserved primer region outside for amplification by PCR. The library can be used directly for the selection of DNA or RNA aptamers. For the selection of RNA aptamers the library should be converted into an RNA library. In the first SELEX round the library and the target molecules are incubated for binding. Unbound oligonucleotides are removed by several stringent washing steps of the binding complexes. The target-bound oligonucleotides are eluted and subsequently amplified by PCR or RT-PCR. A new enriched pool of selected oligonucleotides is generated by preparation of the relevant ssDNA from the PCR products (DNA SELEX) or by *in vitro* transcription (RNA SELEX). This selected oligonucleotide pool is then used for the next selection round. Generally, 8 to 20 SELEX rounds are needed for the selection of high affinity and target-specific aptamers. The last SELEX round is finished after the amplification step. The enriched aptamer pool is cloned and many individual aptamers are then characterized.

Aptamers have a wide range of applications including in medical and pharmaceutical research as well as clinical diagnostic and therapy (77-79). The first FDA approved aptamer with therapeutic function is the anti-human VEGF aptamer (VEGF stands for vascular endothelial grow factor) (80). Pegaptanib is the PEGylated form of this aptamer that is used for the treatment of wet age related macular degeneration. Moreover, research on aptamers for the application in medicine is quite advanced for tumor imaging and therapy, (81,82), and as clinical

diagnostics (e.g., DNA aptamer against Platelet-derived growth factor (PDGF) for the detection of cytokine (83)). They compete with antibodies in adaptability and range of applications (84,85). They are relatively small, are chemically synthesized and pose little to no immunogenicity (e.g., Macugen, in the phase II study reported no serious side effects resulting from its multiple intravitreal injections (76,86)). Aptamers possess remarkable specificity. They are able to distinguish their targets discreetly by very small structural differences such as the presence or absence of a methyl or a hydroxyl group (87-89). Aptamers may show higher specificity and affinity better than antibodies (87). This high specificity is achieved by the selection process in the SELEX cycles that eliminates sequences binding to closely related analogs of the target.

1.6 Immunoglobulin E

Immunoglobulin E (IgE) is one of five isotypes of human immunoglobulins (antibodies) (IgG, IgA, IgM, IgD, and IgE). IgE is produced by B cells and plasma cells like all other immunoglobulins and is found in the lungs, skin and mucus. All the immunoglobulins have a common structure of four peptide chains that consists of two light chains and two identical heavy chains. The heavy chain differentiates between the different immunoglobulin isotypes (Figure 1-5).

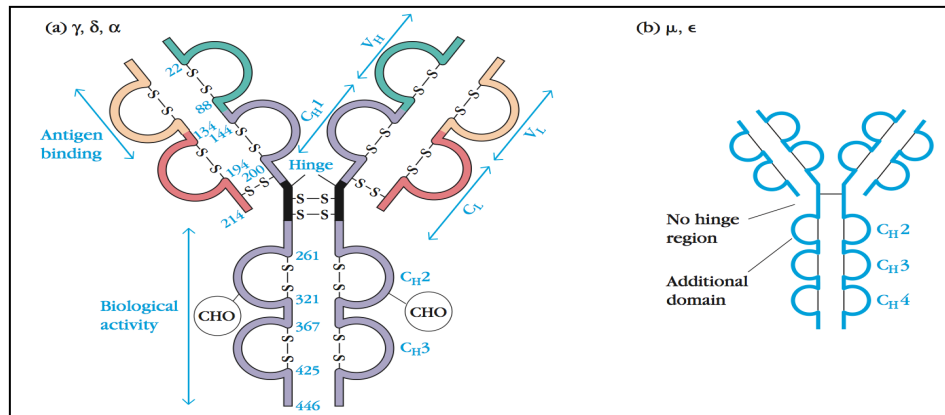


Figure 1-5: General structure of immunoglobulins showing the heavy and light chains in different isotypes (Adapted from Kuby Immunology, 7th edition).

The heavy chain in IgE is ϵ (epsilon). IgE exists as a monomer and has an extra domain in the constant region (CH4) (total four constant domains), in contrast to other immunoglobulins that contain only three constant regions (Figures 1-5 and 1-6).

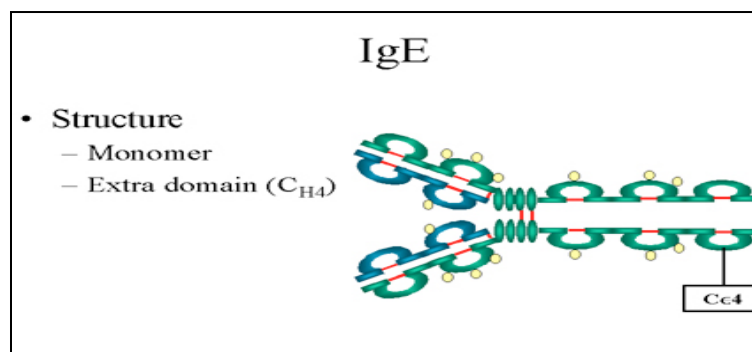


Figure 1-6: Structure of Immunoglobulin E monomer showing the extra domain (source: <http://pathmicro.med.sc.edu/mayer/igstruct2000.htm>)

The molecular weight of IgE is 190 kDa. It is the only immunoglobulin isotype to contain inter-heavy chain disulfide linkages on either side of the CH₂ domain. IgE is found at the lowest levels of all human immunoglobulin isotypes in human serum (0.0005% of total adult serum immunoglobulin levels in nonatopic individuals) and in adults usually are < 200 IU/mL (<480 µg/L) (90). Despite its extremely low serum concentration, it was identified in the serum due to its dynamic biological activity. The IgE antibodies are responsible for mediating the immediate hypersensitivity reactions that induce the symptoms of dermatitis, hay fever, allergic asthma, hives and anaphylactic shock. It is capable of triggering the most powerful immune reactions even though it is the least abundant antibody. Due to the presence of the extra constant domain, IgE possesses a special property of binding to the surface of mast cells and basophils.

The development of the IgE antibody-mediated immune response in allergy begins with sensitization. During the sensitization phase, plasma cells produce IgE antibodies, which bind to the IgE receptors on the surface of basophils in the blood and mast cells in the connective tissue of the skin, respiratory and digestive tracts, and around the vessels. The second exposure to an allergen in the sensitized individuals initiates an aggressive response. The IgE antibodies cross-link with the allergen, which induces basophils and mast cells to translocate their granules to the plasma membrane and release the contents to the extracellular environment. This process known as degranulation release various pharmacological mediators (e.g., histamine, platelet activating factor, neutrophil chemotactic factor of anaphylaxis,

leukotrienes). These mediators cause the dilation of small blood vessels, constrict smooth muscle in pulmonary blood vessels, and increase in vascular permeability and post-capillary leakage, mucous secretion, and other post inflammatory activities. And this ultimately, leads to immediate type I hypersensitivity response, that ranges from localized cutaneous swelling and erythema to more systemic broncho-constriction, angioedema and, in severe cases, anaphylaxis and death (91). IgE is also implicated in parasitic infections apart from allergic reactions (92).

Prevalence of allergic diseases and elevated IgE: Individuals with allergic atopic conditions (e.g., atopic dermatitis, asthma, and allergic rhinitis) are characterized with markedly elevated levels of IgE antibodies (93). The levels increase up to 10 times the normal level of IgE in their blood in allergic individuals. Elevated levels of IgE have been identified as a risk factor even in non-allergic asthma. Studies have shown that, exposure to allergen and subsequent production of IgE are associated with an increased chance of development of asthma within atopic families (94). In another study, it has been shown that food allergen triggers the production of IgE in atopic dermatitis and that correlates with the development of skin disease (95). These studies suggest that IgE is a key factor in triggering the allergic inflammation in the skin and airways. Each individual has different IgE antibodies, and each allergic substance (dust mite, cat, grass or ragweed pollen, etc.) stimulates production of its own specific IgE. About 15 to 25% of the population is affected by atopic allergic diseases (96). The prevalence of atopy and asthma has increased in

all developed countries. For example in the United States, a 75% increase in prevalence has been reported since 1980. Therefore, a reliable and accurate assessment of IgE antibodies is critical for patients with atopic allergy from a clinical perspective. Determinations of specific IgE antibodies and its causative allergens can be used to establish the diagnosis of allergic disease and to make treatment decisions.

1.7 Outline of the present work

As mentioned above the sensitivity of conventional LFAs lag behind the elaborate laboratory methods such as ELISA and PCR. While the LFAs are widely useful method for point-of-care diagnostics, there is a felt need for improved LFA analytical sensitivity. This has been addressed in this work, by developing improved reporter particles. In order to obtain a stronger signal per individual affinity agent, viral nanoparticles are employed as the scaffold for attached multiple reporter enzymes chemically. The central goal of the work presented in this dissertation is to employ M13 bacteriophage as a scaffold for multiple enzyme reporters' and anti-analyte antibodies for attachment in lateral flow assays to develop a highly sensitive and specific detection assay for viruses and proteins.

The second chapter describes the use of functionalized viral nanoparticles as ultrasensitive reporters in lateral flow assays for the detection of viruses. The model analyte used in this assay is the *E. coli* bacteriophage MS2 that has been widely used

as a model for viral pathogens. The filamentous M13 bacteriophage is introduced as a reporter molecule where two different approaches have been employed to manufacture antibody and enzyme-doubly-modified M13 bacteriophage used in LFAs. In the first approach, horseradish peroxidase (HRP) and target specific antibodies are chemically attached to the abundant coat protein (pVIII) of M13. The second approach employs SAM-AviTag phage on which biotinylated antibodies are attached by avidin-biotin coupling (23-25). It will be shown that both approaches are suitable for the attachment of intact antibodies; this makes this approach superior compared to the use of single chain fragment variable (scFvs), which often have lower affinity than the parent antibody and which, more importantly requires cloning. These two different M13 phage constructs decorated with peroxidase enzymes and anti-analyte antibodies serve as reporters in LFAs of greatly enhanced sensitivity. The classical approach to LFA that utilizes gold nanoparticles as reporters has been compared with the newly developed assay and has been found to be almost one thousand-fold less sensitive with the same antibodies.

Using M13 bacteriophage as scaffolds for modification, a novel method of protein detection was developed employing DNA aptamers as recognition element. Aptamers are short, functional nucleic acid molecules isolated from large random libraries through a process termed systematic evolution of ligands by exponential enrichment, or SELEX (74,75). The idea was to make an apta-phage construct that could be used to detect protein with great sensitivity. Aptamers have proven to be efficient molecular tools in the field of analytical chemistry. Compared with

conventional biomolecular ligands such as antibodies, aptamer-based ligands exhibit properties such as high affinity, chemical stability and high flexibility and low production cost that make them superior over antibodies. For building the apta-phage construct, DNA aptamers specific to HyHEL 5 (IgG) were screened.

In the next chapter, aptamers and our preliminary work with them are introduced. The screening of DNA aptamers against murine IgG (HyHEL 5) using SELEX is described. The resulting enriched DNA aptamers are characterized for binding to IgG via SPR (surface plasmon resonance) studies and ELONA (Enzyme Linked Oligonucleotide Assay). Since the selected DNA aptamers did not show successful binding to the target in the above-mentioned studies, a well-studied DNA aptamer (IgE aptamer) against IgE was employed in the apta-phage studies. DNA aptamers against human IgE were developed by Wiegand *et al.* (97), a known biomarker associated with atopic allergic diseases on (93). Since the discovery of the IgE aptamer, several aptamer based assay methods have been reported for the detection of IgE (98,99).

However, a lateral flow based assay using apta-phage as reporters has not yet been reported. Immunoglobulin E is implicated in allergic reactions and widely reported as marker of atopic diseases. Therefore a rapid detection of IgE levels in serum samples will be useful for patients afflicted with allergy-mediated disorders. The fourth chapter of this dissertation is focused on application of IgE aptamers in lateral flow diagnostic systems using M13 bacteriophage as scaffolds for modification. The construction of apta-phage utilizing biotin-avidin interactions has

been described. The binding of IgE aptamer to the IgE protein is validated by ELONA. Finally, the lateral flow based assay for detection of IgE is shown in direct and sandwich assay formats.

Chapter 2: **FUNCTIONALIZED VIRAL NANOPARTICLES AS
ULTRASENSITIVE REPORTERS IN LATERAL-FLOW ASSAYS**

2.1 Introduction

Bacteriophage are commonly used in screening for the identification of protein-ligand interactions (56,100) including interactions between peptides and various cellular proteins (101), and peptides with high binding affinity to inorganic compounds (102). Phage display technology has been utilized for various applications including selecting M13 mutations for diverse applications such as high-power phage batteries (103,104) tissue regenerating materials (105), targeted cancer therapies (106), biological sensors and cell-targeting agents (107-109), and gene transfer vectors (110,111).

Going beyond traditional phage display, bacteriophage have been known to take new roles as building blocks to generate highly sophisticated structures useful in diverse applications including diagnostics (18). They have gained huge interest in the scientific community due to their potential in a variety of applications such as in drug delivery (103-105) as *in vitro* imaging scaffolds (106,107) and as platforms for biosensing (108-110). They pose several advantages that make them useful scaffolds for imaging and detection. For example, they occur naturally and are readily produced by *E. coli*, which enables the generation of many virus copies without intensive fabrication. In addition, their short replication time helps to generate large quantities of any given bacteriophage-based reagent. They are thermally and chemically stable (103) and their protein coat can be modified chemically or genetically, providing an inherent capacity for multivalent and

orthogonal display. In this aspect, M13 bacteriophage have proved to be a promising virus scaffold due to their well-established role in phage display technologies (112). M13 phage is a rod-like virus consisting of a circular, single-stranded DNA enclosed by 2700 copies of the major coat protein pVIII and capped with 5 copies of four minor coat proteins (pIX, pVII, pVI, and pIII) on the ends (Figure 1-3). The minor coat proteins are involved in biological recognition and the major coat protein pVIII function in the structural arrangement of bacteriophage (113). The structure of the phage allows for the major coat proteins to be chemically modified without disrupting its ability to bind a particular antigen. Recently thiol-modified M13 bacteriophage were produced that were able to aggregate gold nanoparticles and the resulting plasmon resonance shifts acted as optical indicators of antigens in solution (114).

Many of the basic protein bio-conjugation techniques permeate fundamental virus chemistry, targeting available amino acids, such as lysines, glutamic or aspartic acids, and cysteines with a variety of molecules, such as enzymes, fluorescent dyes and as quantum dots (115). For M13 bacteriophage, it has been shown that the majority of the modifications take place at Lys8, which is exposed to the exterior environment. There are five lysines (Lys8, 40, 43, 44 and 48) on single coat protein for reactive primary amine, but among them, only one lysine (Lys8) is easily accessible [9]. M13 bacteriophage have been chemically functionalized with drugs (116,117), near-infrared fluorescent dyes (118,119), carboxylic acid groups (120), and various fluorophores (121,122). Li *et al.* (123) synthesized dual-modified

M13 particles with folic acid and fluorescent molecules that could target HeLa contaminant KB cell line and emit fluorescence upon target binding. The pVIII coat protein was modified for the surface expression of folic acid. The lysine groups and N-termini on the bacteriophage surface were conjugated to carboxylic acid and glutamic acid groups on fluorescent molecules. It was determined that approximately 190 fluorescent molecules were conjugated onto each pVIII subunit, effectively increasing detection sensitivity. These examples show that the pVIII coat protein of M13 bacteriophage could be easily modified using simple bio-conjugation methods.

Phage nanoparticles present versatile scaffolds, with high colloidal stability and storage stability, that are able to integrate a wide range of recognition (antibodies and aptamers) and reporter (enzymes) elements leading to universal, ultrasensitive, bio-detection reporters. We prepared two variants of M13 phage particles functionalized with enzyme (HRP) on the major coat protein. In one set, target specific antibodies were functionalized on the gpVIII coat protein along with HRP. In the other set, target specific antibodies were bound on the gpIII protein of AviTag phage via avidin-biotin linkage. These modified M13 particles were used as reporters in the LFAs that were able to detect MS2 virus with great sensitivity and specificity.

2.2 Materials and Methods

2.2.1 Materials

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (St. Louis, MO). AviTag bacteriophages were a gift from Prof. Brian Kay at the University of Illinois at Chicago. MS2 bacteriophage and its host strain *E. coli* (# 15597) were obtained from the American Type Culture Collection (Manassas, VA). Rabbit anti-MS2 polyclonal antibody was purchased from Tetracore, Inc. (Gaithersburg, MD).

2.2.2 Culture and titration of MS2 phage

For MS2 propagation, the *E. coli* host strain (# 7100) was propagated in tryptic soy broth (TSB) at 37 °C with shaking until log phase. For phage enrichment, 75 µL of bacterial host culture and 100 µL of diluted MS2 stock were added to a sterile tube containing 5 mL of 0.7% tryptic soy agar (TSA). The contents of the tube were gently mixed, and then carefully poured onto a pre-warmed 1.5% TSA plate. After overnight incubation at 37 °C, phage were harvested by gently scraping the top layer of soft agar into a 50 mL tube. Equal volumes (~20 mL) PBS and chloroform were added and the mixture was vortexed for 5 min. After 30 min centrifugation at 4,000x g the MS2-containing aqueous phase was carefully removed and filtered through sequentially smaller low protein-binding filters (0.45/0.22 µm). Aliquots of the MS2 stocks were stored at -80 °C.

The titer of the MS2 bacteriophage was determined by the double agar overlay method. For this, *E. coli* host strain 7100 was grown to mid log phase. Then

75 μ L of these mid-log phase bacteria and 100 μ L of MS2 stock, serially diluted in PBS, were added to a sterile tube containing 5 mL of 0.7% TSA. The contents were mixed and then poured onto a petri dish containing 15 mL of 1.5% TSA. The plates were then inverted and incubated at 37 °C for 16-18 hr. Titers were obtained by counting the plaques and correcting for dilutions.

2.2.3 Culture and titration of AviTag M13 bacteriophage

For M13 bacteriophage propagation, TG1 strain of *E. coli* was grown on M9 plate overnight. A single colony of the TG1 bacterium was inoculated in 5 mL of LB medium at 37 °C until mid-log phase. This culture was infected with a single plaque of AviTag phage and grown for 2 hr at 37 °C. The infected culture was then transferred to a 500 mL 2xTY medium in a 2 L flask and grown overnight at 37 °C. Next day the phage were harvested by centrifugation and discarding the pellet. The supernatant was passed through 0.45 μ m filter to remove any bacteria. The phage was purified by precipitating with 20% PEG in 2.5 M NaCl. After PEG precipitation the pellet was suspended in 1 mL of PBS. Finally 25 μ g/ μ L of ampicillin and 0.02% of azide to the final were added to the prepared phage.

Titration of the phage by plaque assay was done to determine the number of phage per mL. For the titration, *E. coli* host strain TG1 was grown to mid log phase. 300 μ L of the bacterial host and 10 μ L of serially diluted phage were mixed and incubated at 37 °C for 15 min without shaking in 50 mL tubes. 40 μ L of 40 mg/mL X-

Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) and 9 μ L of 500 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) were added to each cell suspension tube. Then 5 mL of 0.8% LB agar was added to each tube and the contents were gently mixed by rolling the tubes between the palms and plated onto agar plates. The plates were incubated at 37 °C overnight. Plates were screened for blue plaques.

2.2.4 Enzymatic biotinylation of M13 bacteriophage

SAM-AviTag phages were enzymatically biotinylated using *E. coli* biotin ligase (Avidity, Aurora, CO, or prepared in-house). For the in-house preparation of the biotin ligase, it was expressed and purified from *E. coli*, harboring pET14v_SUMO-birA, a gift from Prof. Brian Kay, as follows.

2.2.4.1 Biotin ligase expression and purification

Biotin ligase expression was done using the Overnight Express Autoinduction System 1 (Novagen). The method is based on media components that are metabolized differentially to promote growth to high density and then automatically induce protein expression from *lac* promoters. The enzyme was expressed as inclusion bodies. The media components (OnEx solution 1, OnEx solution 2 and OnEx solution 3) from the Overnight Express Auto induction System 1 kit were added to LB medium. The medium was inoculated with a single colony (*E. coli* BL21 cells with birA clone) and grown for ~16 hr at 37 °C. The cells were then harvested

by centrifuging for 45 min, 3,000 x g at 4 °C and pellets washed in 50 mL TBS (20 mM Tris-HCl, pH 8, 150 mM NaCl). The cells were centrifuged again (45 min, 3,000 x g, at 4 °C) and resuspended in 150 mL resuspension buffer (20 mM Tris-HCl, pH 8, 10 mM β -mercaptoethanol). Thereafter 0.1 mg/mL Lysozyme (Sigma-Aldrich) and 1.25% (v/v) Serratia Nuclease Cocktail, an endonuclease from *Serratia marcescens* (Sigma-Aldrich) were added, and incubated for 1 hr at 4 °C. The cells were sonicated for 5 min, and 2 mL 70% NP-40 solution, and 40 mL B-PER (Thermo-Fisher, Waltham, MA) were added. After 1 hr at 4 °C, the cell lysate was centrifuged (30 min, 16,000 x g, 4 °C), and the pellet containing the inclusion bodies was denatured using 7.5 M urea, 0.4 M L-arginine, 10 mM DTT, 50 mM Tris-HCl, pH 8, 500 mM NaCl. The inclusion bodies were refolded in 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5% (v/v) glycerol, 5% (v/v) sucrose, and applied to a chelating Sepharose column charged with 0.1 M NiSO₄. The hexahistidine-tagged biotin ligase was eluted using an imidazole gradient (0.02-0.25 M). Finally the peak fractions were checked for homogeneity using SDS-PAGE.

2.2.4.2 Phage biotinylation reaction

For the biotinylation reaction 100 μ L of 1×10^{11} phage/mL were mixed with 14.3 μ L Biomix-A (0.5 M bicine buffer, pH 8.3), 14.3 μ L Biomix-B (100 mM ATP, 100 mM Magnesium acetate (MgOAc), 500 μ M d-biotin), 10 μ L D-biotin (500 μ M), (Avidity, Aurora, CO), and 1 μ L biotin ligase (2 mg/mL) in 860.4 μ L of Tris-HCl, pH 8 (total

reaction volume 1 mL) and incubated for 1 hr at 25 °C. After one hr the phage were precipitated by adding 200 µL of 20% PEG/2.5 M NaCl to the biotinylated phage reaction. Thereafter, it was incubated on ice for 1 hr and then centrifuged at 11,000x g for 20 min. The phage pellet was resuspended in PBS. To check the efficiency of the biotinylation reaction, ELISA with biotinylated AviTag phage utilizing streptavidin-coated microtiter plates from StreptaWell High Bind (Roche Applied Science, Indianapolis, IN) was done.

Briefly, 100 µL of several phage dilutions ranging from 1×10^5 to 1×10^{10} phage/mL were added to streptavidin pre-coated wells and incubated on an orbital shaker for 1 hr at 25 °C. The wells were then washed three times with 300 µL PBS on a Tecan Hydroflex microplate washer to remove any unbound phage. Thereafter 100 µL of anti-M13 antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare Life Sciences, Pittsburgh, PA) was added (1:5,000 diluted) to each well and incubated for 1 hr on a shaker at 25 °C. The wells were again washed three times with 300 µL PBS and then 100 µL 1-Step™ Ultra TMB-ELISA Substrate (Thermo Scientific) was added to each well. The reaction was stopped after 20 min with 50 µL 2 M H₂SO₄ and absorbance was measured at 450 nm in a Tecan Infinite M200Pro microplate reader.

2.2.5 Chemical biotinylation of anti-MS2 antibody

Rabbit anti-MS2 polyclonal antibody was purchased from Tetracore, Inc. (Gaithersburg, MD), and biotinylated using EZ-Link Sulfo-NHS- LC-Biotin (Thermo Fisher Scientific) reagent using a 20-fold molar excess of biotin reagent according to the manufacturer's instructions. Briefly, 1 μ L of 2.8 mg/mL anti-MS2 antibody was mixed with 2 μ L of 0.1 mg/mL of EZ-Link Sulfo-NHS- LC-Biotin (20 molar excess than antibody) in 117 μ L of PBS, pH 7.4 for 1 hr at room temperature. After the incubation reaction mixture was passed through Zeba™ spin desalting columns (7KDa, MWCO) to remove the excess biotin. Extent of biotinylation was estimated using the HABA reagent (Thermo Scientific). The number of biotin molecules per antibody was calculated using a formula provided by supplier as shown below.

1) Concentration of biotinylated protein in mmol/mL [#1]

$$\text{mmol biotinylated antibody/mL} = \frac{\text{antibody concentration (mg/mL)}}{\text{MW of antibody (mg/mmol)}}$$

2) Change in absorbance at 500nm [#2]

$$\Delta A_{500} = A_{500} \text{ H/A} - A_{500} \text{ H/A/B}$$

where, $A_{500} \text{ H/A}$ = Absorbance at 500nm for HABA/avidin reaction mixture and
 $A_{500} \text{ H/A/B}$ = Absorbance at 500nm for HABA/avidin/biotin reaction mixture

3) Concentration of biotin in mmol per mL of reaction mixture [#3]

$$\frac{\text{mmol biotin}}{\text{mL reaction mixture}} = \frac{\Delta A_{500}}{34,000 \times b}$$

(b=path length; extinction coefficient of HABA/avidin = $34,000 \text{ M}^{-1}\text{cm}^{-1}$)

4) mmol of biotin per mmol of protein [#4]

$$\frac{\text{mmol biotin}}{\text{mmol antibody}} = \frac{\#3 \times 10}{\#1} (\text{dilution factor}) = (\text{average \# of biotin molecules per antibody})$$

Box 2-1: Calculation of Moles of Biotin per Mole of Protein (adapted from Thermo Scientific)

2.2.6 Different types of Phage modification

The M13 phage coat protein gpVIII provides abundant binding sites for reporters and anti-analyte antibodies. Furthermore the gpIII protein of M13 phage offers binding sites for anti-analytes via avidin-biotin linkage (Figure 2-4 depicts the schematic of M13 phage functionalization using each approach). The approaches were compared for the development of improved reporter particles, the covalent linkage of the antibodies to the phage coat proteins and the avidin-mediated

coupling of biotinylated antibodies to enzymatically biotinylated AviTag peptides displayed on the phage tail proteins

2.2.6.1 Bacteriophage M13 functionalization through avidin-biotin

SAM-AviTag phages were enzymatically biotinylated using *E. coli* biotin ligase (Avidity, Aurora, CO, or prepared in-house) as described in Section 2.2.5. The biotinylated phages were then covalently coupled to horseradish peroxidase (HRP, Sigma-Aldrich) using Traut's reagent (2-Iminothiolane-HCl, Thermo Fisher Scientific, Waltham, MA) and sulfo-SMCC (Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate; Thermo Fisher Scientific). Traut's reagent reacts with the primary amines on the proteins to introduce sulfhydryl groups (Figure 2-1). These sulfhydryl groups can then be targeted for reaction in a variety of crosslinking and immobilization procedures. Sulfo-SMCC is an amine-to-sulfhydryl cross linker that contains amine-reactive N-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group (Figure 2-2). NHS esters react with primary amines to form amide bonds and maleimide reacts with sulfhydryl groups to form stable thioether bonds. The biotinylated phages were reacted with Traut's reagent to introduce sulfhydryl groups while HRP was reacted with Sulfo-SMCC to generate maleimide-HRP. Finally this maleimide-HRP was mixed with Traut's reagent activated phage to form a stable complex via thioether bond (Figure 2-3).

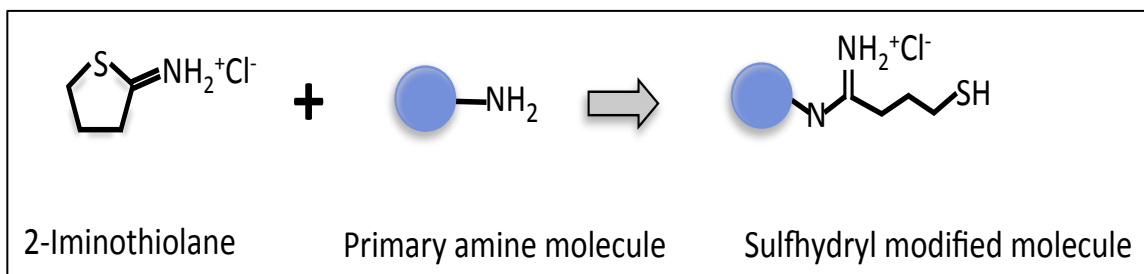


Figure 2-1: Reaction scheme of Traut's reagent (2-Iminothiolane-HCl) for the thiolation (sulfhydryl group addition) of proteins

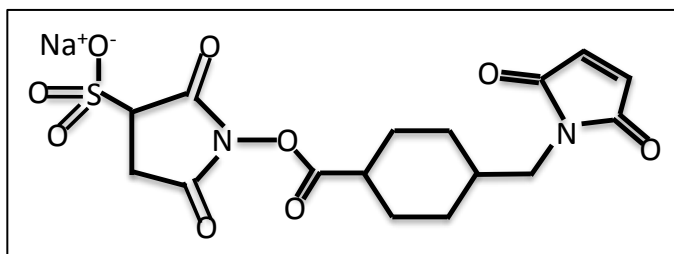


Figure 2-2: Sulfo-SMCC (Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate)

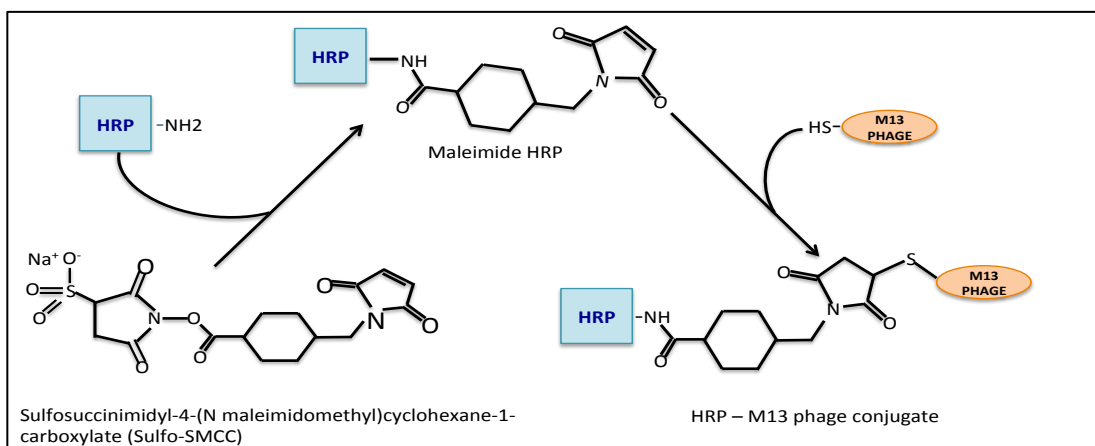


Figure 2-3: Reaction scheme for conjugating HRP and M13 phage with Sulfo-SMCC

Briefly, 100 μL ($\sim 10^{11}$) phage were suspended in 800 μL PBS, 3 mM EDTA (Ethylenediaminetetraacetic acid). EDTA in the buffer is added to chelate divalent metals in the solution, which helps to prevent oxidation of sulfhydryls (i.e., formation of disulfide bonds). Traut's reagent was added to a final concentration of 7 μM and the reaction was allowed to continue for 90 min at 25 $^{\circ}\text{C}$. Excess Traut's reagent was removed using 10 kDa filters (Millipore, Billerica, MA). Maleimide-HRP was separately prepared by mixing HRP and sulfo-SMCC to achieve final concentrations of 22 μM and 1.14 mM, respectively, in 1 mL PBS for 30 min. Excess sulfo-SMCC was removed using a 10 kDa filter. Traut's reagent-modified phage and sulfo-SMCC-modified HRP were then mixed for 90 min at 25 $^{\circ}\text{C}$. Free HRP was removed using a 100 kDa filter (Millipore). Neutravidin (Thermo Fisher Scientific) was bound to the HRP-labeled phage through the biotinylated AviTag displayed on

phage protein III. Rabbit anti-MS2 polyclonal antibody was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) reagent using a 20-fold molar excess of biotin reagent according to the manufacturer's instructions. Neutravidin-functionalized phage and biotinylated antibody (at a 10-fold molar excess) were incubated for 1 hr at 37 °C, before uncoupled antibodies were removed using a 300 kDa spin dialyzer (Harvard Apparatus, Holliston, MA).

2.2.6.2 Bacteriophage M13 functionalization through covalent coupling of antibody and reporter enzyme

Here anti-MS2 antibodies and HRP were chemically coupled on M13 major phage coat protein (Figure 2-4A). M13 phage were activated using Traut's reagent as described in Section 2.2.6.1. Maleimide-HRP was separately prepared by mixing HRP and sulfo-SMCC in a final concentration of 22 μ M and 1.14 mM, respectively in 1 mL PBS for 30 min. Similarly anti-MS2 antibody was also activated using sulfo-SMCC to provide maleimide groups. Excess sulfo-SMCC was removed using a 10 kDa filter. Thereafter Traut's reagent-modified phage, sulfo-SMCC-modified HRP and sulfo-SMCC modified anti-MS2 antibody at a molar ratio (HRP: antibody: phage) of 270,000:10:1 were mixed for 90 min at 25 °C. Excess HRP was removed using 100 kDa filters (Millipore). After 90 min at 25 °C, the complex was dialyzed using a 300 kDa spin dialyzer (Harvard Apparatus, Holliston, MA) to remove free enzyme and antibody.

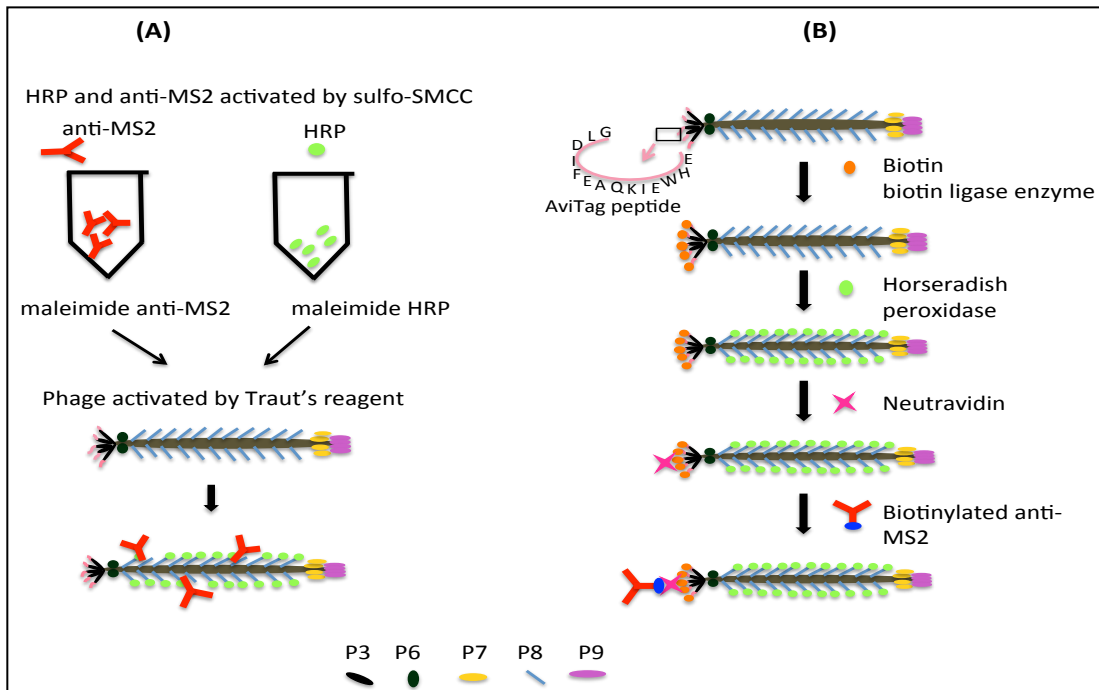


Figure 2-4: Schematic depiction of functionalization of M13 phage

(A) Chemical coupling of HRP and anti-MS2 antibodies on phage coat protein. (B) Coupling of anti-MS2 antibodies on AviTag phage via avidin-biotin linkage.

2.2.7 Real-time PCR of the phage constructs

After the final modifications, the phage constructs were subjected to real-time PCR to obtain the concentration of phage constructs. A series of 10-fold serial dilutions (10^5 to 10^{10} phage/mL) of unmodified phage with known concentration (titer obtained by plaque assay) was used as standards along with the dilutions of phage constructs. The concentrations of phage constructs were determined against the standard curve derived from the unmodified phage standards. The AviTag-targeted

PCR primers were, forward: 5'-GTTGTTTCTTTCTATTCTCACT-3' and reverse: 5'-CAGACGTTAGTAAATGAATTTT-3'. For the phage amplification 0.1 μ L of 10 μ M of each forward and reverse primer were combined with 10 μ L of 2xPCR mix (Brilliant III Ultra-Fast SYBR mix, Agilent, Santa Clara, CA), 4.8 μ L of ultra-pure (RNase and DNase free) water and 5 μ L of each phage sample to achieve a total volume of 20 μ L. The PCR conditions were, 10 min at 95 °C, 40 cycles of 30 seconds at 62 °C and 30 seconds at 72 °C, followed by dissociation step (1 minute at 95 °C, 30 seconds at 55 °C, and 30 seconds at 55 °C).

2.2.8 Lateral flow assay with phage constructs

The model analyte used in the assay was the *E. coli* bacteriophage MS2 that has been widely used as a model for viral pathogens. Serial dilutions of the phage were prepared from a stock whose titer had been determined using the double agar overlay plaque assay, the standard for the enumeration of MS2 (115), and independently confirmed spectrophotometrically (124). About 10^8 modified M13 phage constructs were used to capture MS2 virus on anti-MS2 antibodies spotted on Fusion 5 glass fiber LFA matrix (Figure 2-5 depicts the schematic of phage LFA).

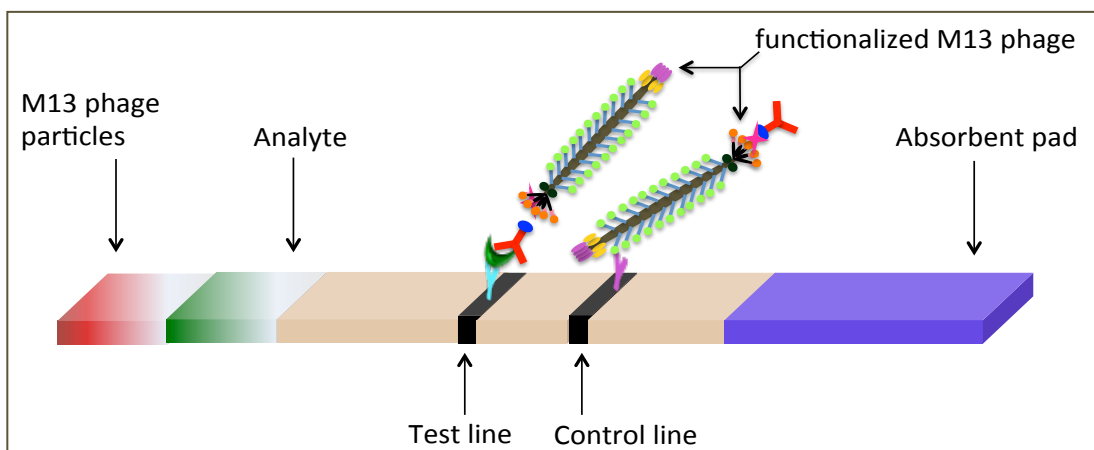


Figure 2-5: Scheme of phage LFA

2.2.8.1 Preparation of LFA strips

To form MS2-detection and M13 control lines, 5 μL anti-MS2 antibodies (0.4 $\mu\text{g}/\mu\text{L}$) and 5 μL anti-M13 antibodies (0.1 $\mu\text{g}/\mu\text{L}$), GE Healthcare, Piscataway, NJ), both in 50 mM sodium acetate buffer (pH 3.6), were spotted on Fusion 5 membranes (GE Healthcare Biosciences, Piscataway, NJ) using a Lateral Flow Reagent Dispenser (Claremont BioSolutions, Upland, CA) equipped with an external syringe pump (Chemyx, Stafford, TX). The strips were allowed to dry for 1 hr at 25 $^{\circ}\text{C}$.

2.2.8.2 Test procedure and measurements

The bacteriophage MS2 test analyte was diluted in LFA buffer (100 mM Tris-HCl, pH 8; 0.3% Tween-20; 0.2% BSA; 0.1% PEG-3350). 100 μL samples were pipetted onto the sample application area of a prepared 7x50 mm Fusion 5 strip with anti-MS2 antibodies on the detection line and anti-M13 antibodies on the control line (Figure

2-5 depicts the schematic of phage LFA). The strips were first washed with 200 μL test buffer before 10 μL of the respective phage construct ($\sim 10^8$) were dispensed onto the distal end of the LFA strip. The strips were washed again with 500 μL LFA buffer. Finally the signals were obtained by spotting 25 μL TMB Liquid Substrate System for Membranes (Sigma-Aldrich, St. Louis, MO) on each line. The strips were scanned on a Perfection V600 flatbed color scanner (Epson, Long Beach, CA).

2.2.8.3 Analyzing the data through ImageJ

To get quantitative information for the results, the LFA strips after were scanned and analyzed by ImageJ's density analysis function. Both the control and test lines were scanned through ImageJ. The intensities of the test line were divided by the control line intensities to adjust for any background. The ratio between the intensity of the test line to the control line was used as the LFA signal. We define the Limit of Detection (LOD) as the first concentration for which the signal is significantly different from the negative control signal.

2.2.9 Comparison of phage LFA with the classical gold nanoparticles LFA

As a comparison with the classical LFA with our phage based LFA, gold-nanoparticle LFA was also performed to detect MS2 virus utilizing the same antibodies. The gold-nanoparticle LFA was performed on the standard nitrocellulose membrane.

2.2.9.1 Preparation of gold nanoparticle reporters

100 μ L of 4 mM K_2CO_3 was added to 1 mL of 40 nm gold nanoparticles (10^{11} /mL, OD₅₂₀=1, DCN, Carlsbad, CA). Then 10 μ g of rabbit anti-MS2 antibodies were added to the gold nanoparticles and were adsorbed for 20 min at 25 °C on a rotator. 100 μ L of BSA (10% (w/v)) was added to block the nanoparticles. After 20 min, the functionalized nanoparticles were collected by centrifugation (5 min, 10000 x g). The particles were washed once with 1 mL of storage solution (PBS, pH 7.4; 1% (w/v) BSA; 10% (w/v) sucrose), thereafter suspended in 100 mL and stored at 4 °C. To estimate the concentration of gold nanoparticles their absorbance at 520 nm was measured.

2.2.9.2 Classical gold nanoparticle LFA

For the gold nanoparticle LFA, nitrocellulose membrane, Prima 60 (GE Healthcare Biosciences, Piscataway, NJ) was used. The test line with anti-MS2 antibodies (1 μ g/cm) and control line with anti-rabbit antibodies (0.25 μ g/cm) were made using Lateral Flow Reagent Dispenser (Claremont BioSolutions, Upland, CA) equipped with an external syringe pump (Chemyx, Stafford, TX). The strips were allowed to dry for 1 hr at 37 °C. For the assay the strips (5 mm x 40 mm) were cut and 20 μ L LFA buffer (100 mM Tris-HCl, pH 8; 0.3% (v/v) Tween-20; 0.2% (w/v) BSA; 0.1%

(w/v) PEG 3350) was added at the bottom of the strip. Thereafter dilutions of MS2 viral particles in LFA buffer were added followed by 60 μ L LFA buffer. Functionalized gold nanoparticle reporters (2×10^9) were added in 20 μ L of LFA buffer, followed by another 60 μ L LFA buffer. The strips were left to dry and thereafter scanned in a Perfection V600 flatbed color scanner (Epson, Long Beach, CA).

2.3 Results and Discussion

2.3.1 Biotinylation of M13 bacteriophage

The extent of biotinylation of AviTag phage was evaluated via ELISA on a streptavidin-coated microtiter plate on which serial dilutions of phage ranging from 1×10^3 to 1×10^{10} phage in 100 μ L of PBS were added and incubated for 1 hr at 25 °C. After washing thrice with PBS, 100 μ L of anti-M13 antibody conjugated to HRP was added to each well and incubated for another hr. The plate was washed thrice before color was developed by adding TMB ELISA substrate. The reaction was stopped with 50 μ L of 2 M H_2SO_4 , and the absorbance was measured in the plate reader at 450 nm. AviTag phage originally are partially biotinylated while grown (25). As shown in Figure 2-6, biotin ligase treated AviTag phage is retained at higher levels on a streptavidin plate confirming the increased biotinylation level compared to that of the original phage (AviTag phage without biotin ligase treatment).

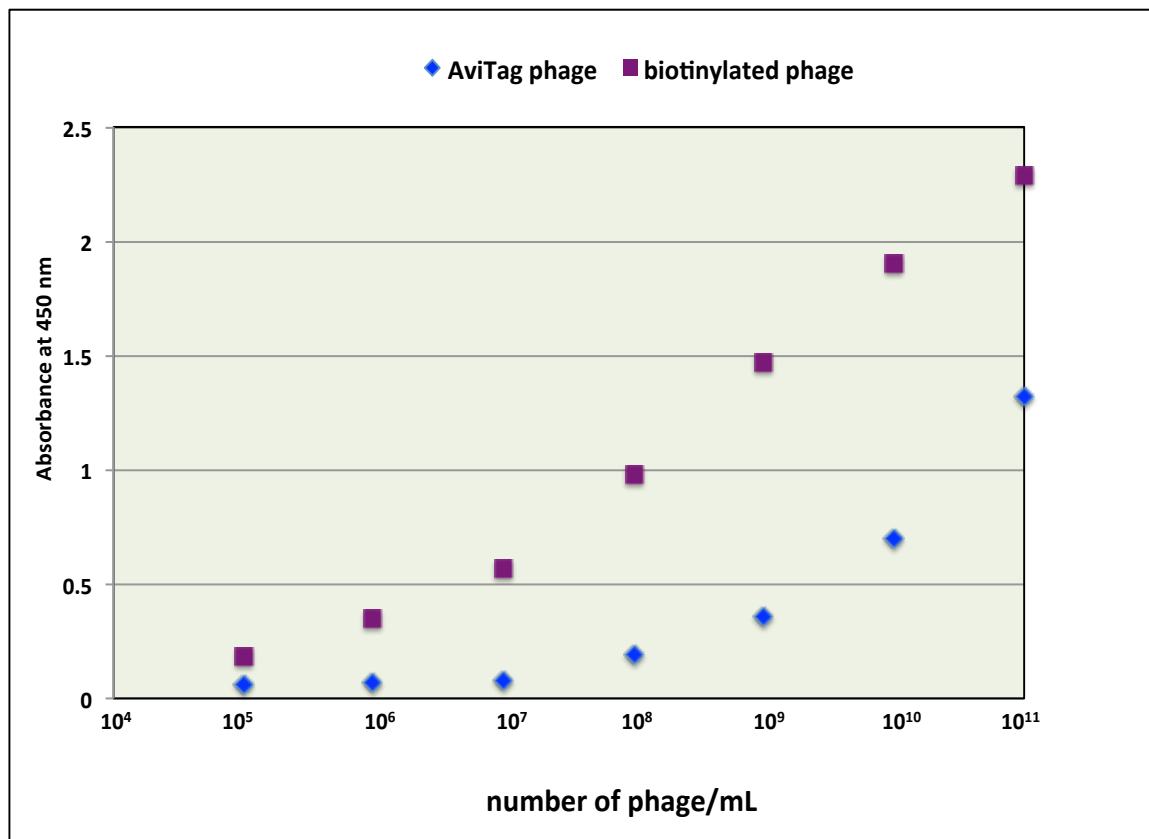


Figure 2-6: ELISA result of enzymatic biotinylation of AviTag phage showing the confirmation of biotinylation of AviTag phage by enzymatic treatment

2.3.2 Biotinylation of anti-MS2 antibodies

The degree of antibody biotinylation was determined using the HABA (4'-hydroxyazobenzene-2-carboxylic acid) assay (HABA-Avidin solution, Thermo Scientific, manufacturer's protocol). HABA is a reagent that enables estimation of the mole-to-mole ratio of biotin to protein. This HABA-avidin complex absorbs at

500 nm. Initially HABA molecules are bound to neutravidin. After being mixed with biotinylated antibody that displaces HABA molecules on neutravidin, the absorbance at 500 nm decreases. Based on the absorbance signal change, the number of biotin molecules per antibody was calculated using a formula provided by supplier (as shown in Section 2.2.5). The calculated number of biotin molecules per antibody was ~ 3 biotins/anti-MS2 antibody.

2.3.3 Determination of M13 phage construct titers by PCR

Real-time PCR was done after every phage modification to monitor the concentration of the phage. The concentration was obtained using a standard curve derived from a set of phage standards with known concentrations whose titer had been obtained by plaque assay. For this, a set of standard curves was prepared for the accurate quantitation of phage. The initial phage stock titer was first determined by the plaque assay in which serial dilutions of phage ranging from 10^4 to 10^{12} -fold were taken and the number of phage in each dilution was determined. Then real time quantitation was done using Brilliant III Ultra-Fast SYBR mix and primers specific for SAM-AviTag phage as described in Section 2.2.7. Figure 2-7 shows a typical amplification plot of M13 phage over 40 cycles. As shown in Figure 2-7, phage amplify well with an average reaction efficiency of $98.4\% \pm 5.5$. A linear regression curve was generated across the phage dilutions ($r^2=0.998$) by plotting the cycle threshold values against $\log_{10}$ phage number. The linear range for phage

detection by real- time PCR was determined from 10^4 to 10^{10} phage/mL or 5×10^1 to 5×10^8 phage per reaction (Figure 2-8). A close correlation was established between real-time PCR signal and the number of phage per reaction that was determined microbiologically (Figure 2-8, $r^2 = 0.998$). The number of functionalized phage/mL was determined from this standard curve. We observed that the phage recovery after the various purification steps via filters and dialysis during the modification was on an average $\sim 90\% \pm 5$.

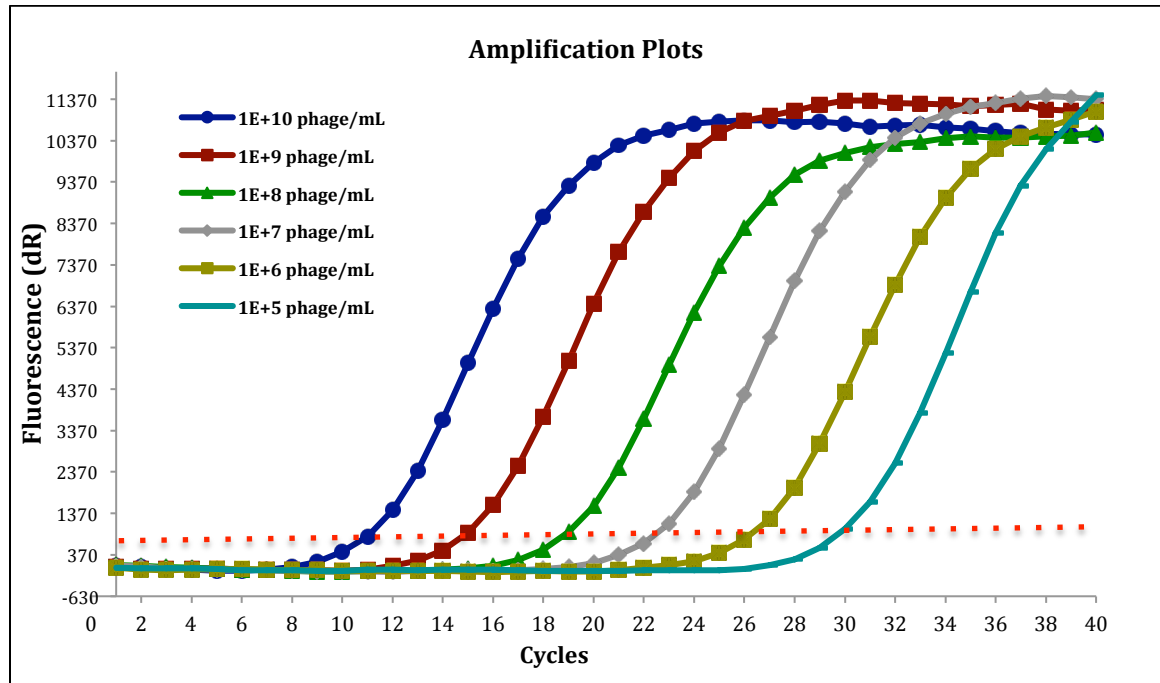


Figure 2-7: Real-time PCR of phage showing the amplification plots of 10-fold serial dilutions of phage (10^4 to 10^{10} phage/mL)

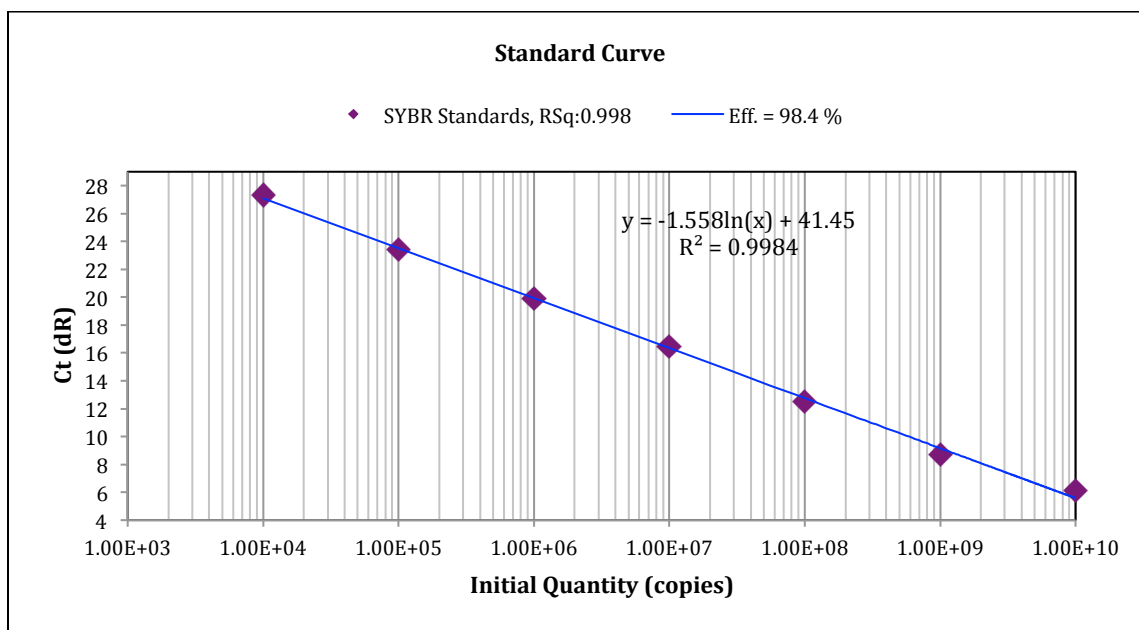


Figure 2-8: Real-time PCR standard curve of AviTag phage showing the correlation of Ct values and phage titers as determined by agar overlay plaque assay

2.3.4 LFA using an antibody-phage construct modified via avidin-biotin coupling

Serial dilutions of the MS2 phage were prepared from a stock whose titer had been determined using the double agar overlay plaque assay, the standard for the enumeration of MS2 (115). Anti-MS2 antibodies were spotted at the detection line to capture any MS2 phage and subsequently any viral nanoparticles with anti-MS2 antibodies. After adding the enzyme substrate, the LFA strips were scanned in a Perfection V600 flatbed color scanner.

As shown in the Figure 2-9, a signal is obtained at the anti-M13 control line for all samples, indicating that the viral nanoparticle reporters successfully moved through the membrane. At the test line, the signal obtained for 10^3 MS2 particles is clearly distinguishable from the control containing no phage (Figure 2-9). Given a sample volume of 100 μ L, this corresponds to a titer of 10^4 pfu/mL. ImageJ density analysis was performed to quantitate the peak areas (Figure 2-9B). The relative intensities of the test and control lines of the strips were also obtained through the ImageJ analysis function (Table 2-1). The relative intensities of the test lines correspond to number of MS2 detected by the phage reporters, and it correlates well with the signals obtained on the strip by simple visual inspection. To adjust for any background variation, relative intensities of the test lines were divided by the control lines (T/C). A close correlation was observed between the T/C values and the MS2 concentration (Table 2-1). The ratio between the intensity of the test line to the control was used as the LFA signal. Based on the ImageJ density analysis function, the limit of detection was 10^4 pfu/mL. This represents an almost 1000-fold increased sensitivity compared to a gold nanoparticle LFA using the same antibodies (Figure 2-12) and 100-fold improvement in sensitivity over previously reported LFAs for viral detection (30,31). As a control, we have shown that without anti-MS2 antibodies the M13 phage is not non-specifically retained by the anti-MS2 antibodies (Figures 2-9 and 2-10).

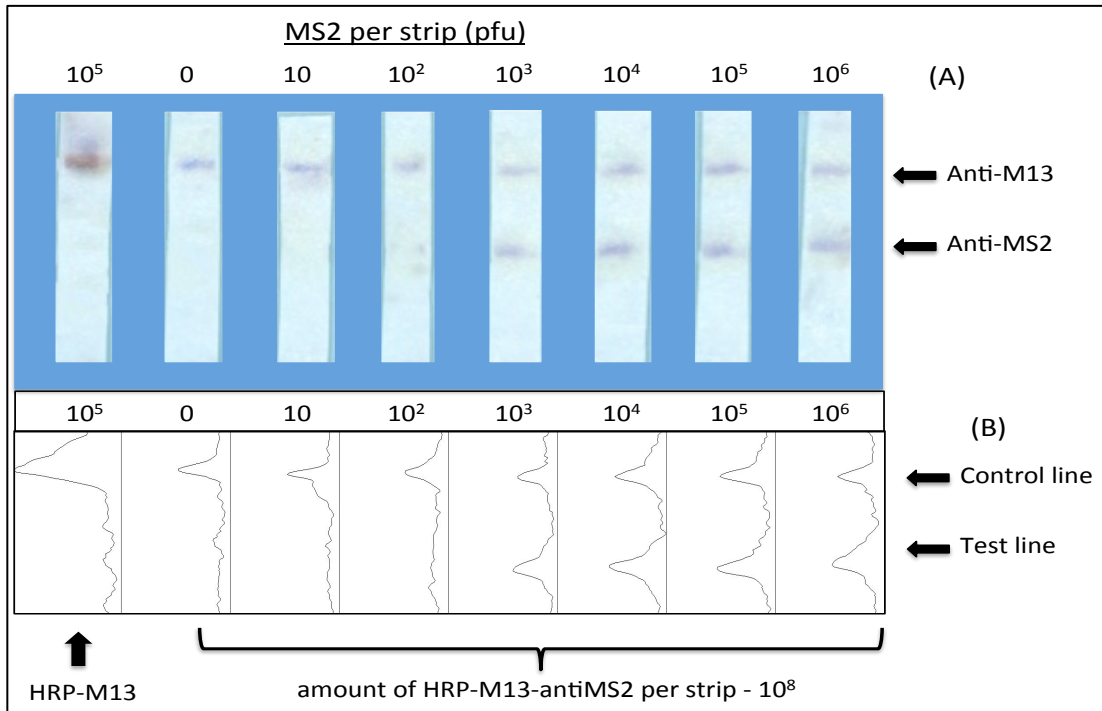


Figure 2-9: Immunochromatographic detection of bacteriophage MS2

(A) Various numbers of MS2 phage were added to a Fusion 5 strip containing anti-MS2 and anti-M13 antibodies. Anti-MS2 antibodies were added to a biotin on the tail protein (pIII) of the AviTag phage via avidin-biotin conjugation. Horseradish peroxidase (HRP) was chemically linked to the coat proteins, gpVIII. About 10^8 of this modified phage was passed on the membrane to bind on test lines (in the presence of target) and control lines. AviTag phage carrying just the HRP reporter (HRP-M13) enzyme were used as a control. (B) Plots of test and control lines of the LFA strips in Figure 2-9A as determined ImageJ density analysis.

Table 2-1: Relative intensities of the test lines and control lines from Figure 2-9 as determined by ImageJ

MS2 phage/mL	10 ⁶	0	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷
Control line	10555	2699	3441	2778	1753	4457	2933	2592
Test line	0	0	0	0	2236	6965	5003	5433
Test/Control	0	0	0	0	1.27	1.56	1.71	2.09

2.3.5 Lateral flow assay by antibody-phage construct modified via covalent coupling

In the alternate approach for preparing phage reporters, HRP and anti-MS2 antibodies were covalently coupled to M13 phage's pVIII major coat protein. LFA with these modified phage was performed to capture MS2 virus similarly as described above in Section 2.3.4. The results of this LFA are depicted in Figure 2-10. A clear signal on the control line confirms that the M13 phage particles move through the membrane and bind on the anti-M13 antibody and hence assay the assay works well. In the test lines, a distinguishable signal from the control containing no MS2 is obtained on the strip with 10³ MS2 /strip. The strip with HRP-phage (M13 phage bearing HRP on the coat but devoid of anti-MS2 antibodies) label did not give any signal, indicating that phage did not bind non-specifically to the anti-MS2 line. The test and control lines were quantified by ImageJ density's analysis function that reveals the limit of detection to be 10⁴ pfu/mL. The plots and

relative intensities of test and control lines are depicted in Figure 2-10 (B) and Table 2-2, respectively.

We did not observe any significant difference in sensitivity between M13 phage containing anti-MS2 antibodies attached to the pIII tail protein, and those with the antibodies attached to the phage coat. Assuming that the number of pIII molecules is between three and five per phage, and the number of pVIII coat proteins is 2700, we would have expected a significantly larger number of antibodies per phage in the latter approach.

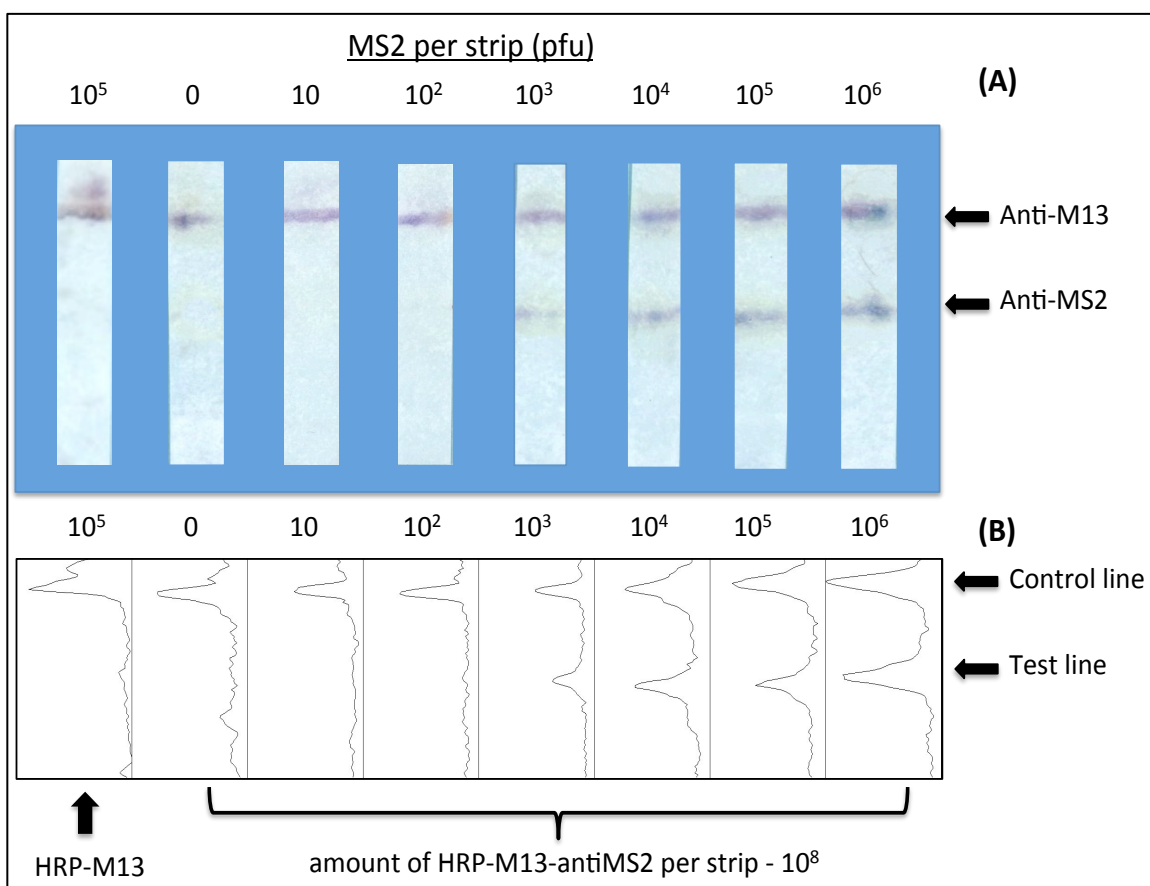


Figure 2-10: Immunochromatographic detection of bacteriophage MS2 by M13 phage functionalized chemically with HRP and anti-MS2 antibodies through covalent binding

(A) Various numbers of MS2 in 100 μ L LFA buffer were passed through the LFA membrane with anti-MS2 antibodies as the test line, and anti-M13 antibodies as the control line. Functionalized M13 phage particles with HRP and anti-MS2 antibodies were added to bind to both the test (in the presence of analyte) and control lines. M13 phage carrying just the HRP reporter (HRP-M13) enzyme were used as a control (B) Plots of test and control lines of the LFA strips in Figure 2-10(A) as determined by ImageJ density analysis.

Table 2-2: Relative intensities of the test lines and control lines shown in Figure 2-10 as determined by ImageJ

MS2 phage/mL	10^6	0	10^2	10^3	10^4	10^5	10^6	10^7
Control line	5877	5816	4354	3762	3852	4657	4888	4358
Test line	0	103	0	39	2510	3975	4045	5482
Test/Control	0	0.02	0	0.01	0.65	0.85	0.83	1.25

2.3.6 Detection of dialyzed MS2 phage as control for phage fragments

To ensure that the MS2 preparation was free of any free phage fragment or any other protein that may lead to non-specific binding or increase the effective number of targets in the assay and interfere with the results, MS2 was dialyzed using a 300 kDa spin dialyzer. The dialyzed MS2 was then assayed for detection by the functionalized phage constructs. Figure 2-11 shows the results for detection of dialyzed MS2 phage. In this control experiment, the limit of detection was again observed to be 10^4 pfu/mL. The data was quantified with ImageJ analysis function. As depicted in Figure 2-11 and Table 2-3, results of the ImageJ analysis density function also correlates well with the visual inspection of the strips.

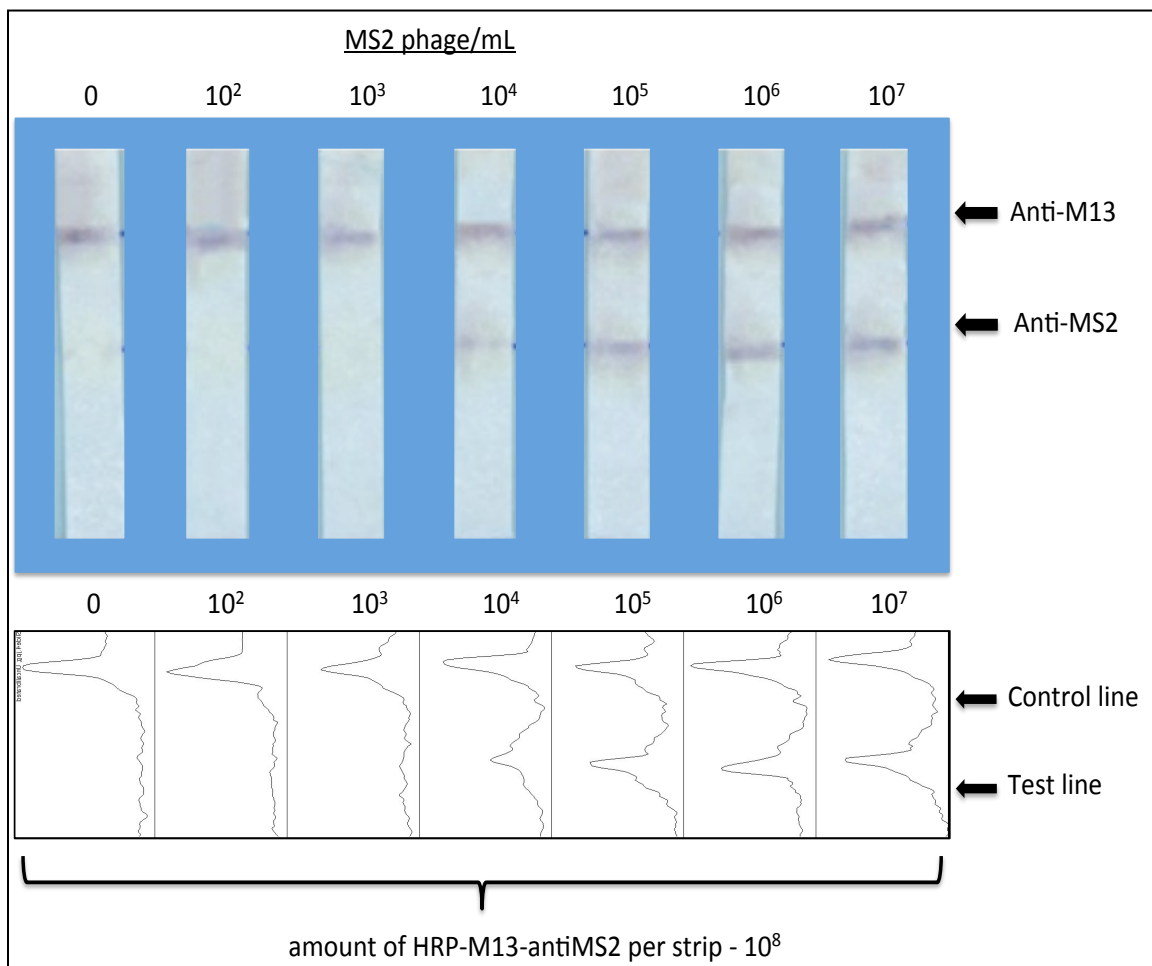


Figure 2-11: Immunochromatographic detection of dialyzed MS2 phage. MS2 phage was dialyzed using 300 kDa spin dialyzer

(A) Various numbers of this dialyzed MS2 phage in 100 μ L LFA buffer were added to a Fusion 5 strip containing anti-MS2 and anti-M13 antibodies. About 10⁸ M13 phage reporter particles were added on each strip to bind on test lines (in the presence of target) and control lines. (B) Plots of test and control lines of the LFA strips in Figure 2-11(A) as determined by ImageJ density analysis.

Table 2-3: Relative intensities of the test lines and control lines shown in Figure 2-11 as determined by ImageJ

MS2 phage/mL	0	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷
Control line	6767	5980	5977	5574	4997	5435	4803
Test line	94	0	100	4757	5346	5985	7590
Test/Control	0.01	0	0.01	0.85	1.06	1.10	1.58

The success of M13 reporters with low antibody loads is consistent with the successful practice of phage-display library screening in the monovalent phage display format, in which a phage is used to display only a single genetically encoded candidate antibody on the M13 tail protein, eliminating the confounding effects of multivalent binding by multiple copies of the displayed antibody (56,100). The success of this method implies that phage are retained at significant efficiency by affinity of a single displayed antibody, and argues that M13 LFA reporters should display small numbers of antibodies and a maximal number of enzyme reporters.

2.3.7 Reproducibility of the assay

The reproducibility of the experiments was analyzed by repeating the MS2 phage detection assay by phage functionalized with both approaches three times. Relative intensities of the test lines for six independent lateral flow assays are shown in Table 2-4. As shown in the table 2-4, they show identical detection limits (Table 1).

Table 2-4: Relative intensity of the test-line for six independent lateral flow assays using functionalized M13 bacteriophage to detect MS2 virus

Number of phage per strip	CC-1	CC-2	CC-3	AV-1	AV-2	AV-3
0	0	0	0	319	0	0
10	0	0	0	180	0	0
1.00E+02	0	0	0	325	233	318
1.00E+03	4613	5374	2100	2470	1541	3235
1.00E+04	7723	6944	3555	5498	3859	6387
1.00E+05	7679	9397	4662	5661	4514	5313
1.00E+06	10441	9004	3856	7818	4341	7486

CC1-3: Reporter enzyme and antibody were covalently attached to M13. AV1-3: the antibody was attached through avidin-biotin linkage and reporter enzyme covalently.

2.3.8 MS2 detection with the classical gold nanoparticles LFA

Dilutions of MS2 viral particles in LFA buffer were added followed by 60 μ L LFA buffer. Functionalized gold nanoparticle reporters (2×10^9) were added in 20 μ L of LFA buffer, followed by another 60 μ L LFA buffer. The strips were left to dry and thereafter scanned in a Perfection V600 flatbed color scanner. As shown in Figure 2-12, the limit of detection of MS2 phage by the functionalized gold nanoparticles is 10^7 viral particles that correspond to 10^8 MS2/mL.

On the other hand, our phage-based detection has a limit of detection of 10^4 pfu/mL that is 1000-fold more sensitive than classical gold-nanoparticle LFA. This tremendous increase in the sensitivity in our lateral flow assays could be attributed to the fact that use of M13 phage reporters bearing 2700 copies of major coat,

provides a huge number of binding sites for reporters such as horseradish peroxidase, as well as for antibodies specific for the target of interest. The use of M13 nanoparticles as the scaffold for attachment of multiple reporter enzymes chemically is successful in obtaining a stronger signal per individual affinity agent. Even considering mutual steric exclusion, the 2700 pVIII coat proteins of phage M13 likely offer hundreds of binding sites for reporters and antibodies specific for the target of interest. We believe that M13 phage modified with abundant enzymes is capable of generating stronger signal and increasing the sensitivity of LFA.

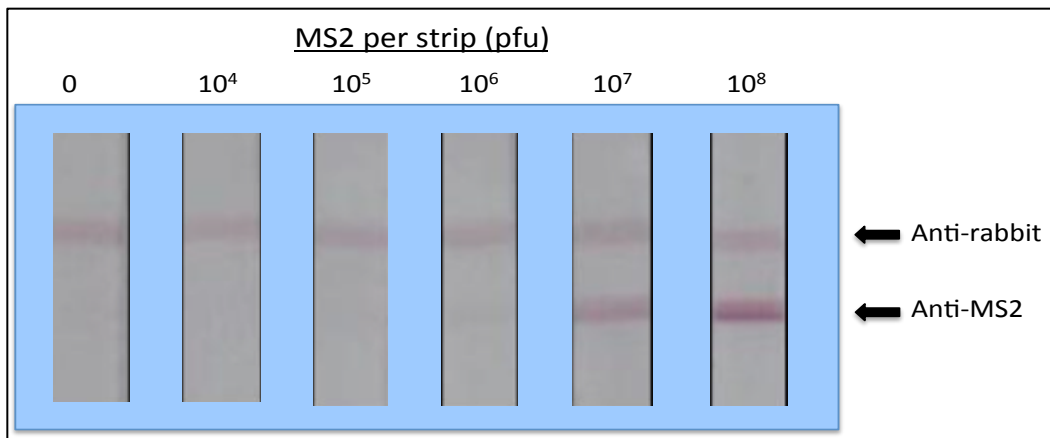


Figure 2-12: Immunochromatographic detection of bacteriophage MS2 using gold nanoparticles as reporters.

Various numbers of MS2 phage were added to a nitrocellulose strip containing anti-MS2 and anti-rabbit antibodies. Gold nanoparticles with adsorbed rabbit anti-MS2 antibodies were captured on the test line by MS2 and on the control line by anti-rabbit antibodies.

2.4 Conclusions

While immunochromatographic assays are a widely useful method for point-of-care diagnostics, there is a need for improved LFA analytical sensitivity. This has been addressed by pre-concentrating the target analyte (17), or, as described in this chapter, by developing improved reporter particles. In order to obtain a stronger signal per individual affinity agent, viral nanoparticles were employed as the scaffold to which multiple reporter enzymes were chemically attached. Even considering mutual steric exclusion, the 2700 gpVIII coat proteins of phage M13 offer hundreds of binding sites for reporters such as horseradish peroxidase, as well as for antibodies specific for the target of interest. For the addition of antibodies to these phage affinity agents, we compared two approaches, the covalent linkage of the antibodies to the phage coat proteins and the avidin-mediated coupling of biotinylated antibodies to enzymatically biotinylated AviTag peptides displayed on the phage tail proteins. Both the approaches were able to detect target with detection limit of 10^4 pfu/mL which represents almost one-thousand-fold increased sensitivity compared to gold nanoparticle LFA with the same antibodies.

Thus, we show that M13 bacteriophage decorated with peroxidase enzymes and anti-analyte antibodies can serve as reporters in lateral-flow assays of greatly enhanced sensitivity. We plan to examine the use of alternative enzyme reporters and substrates for our LFA scheme, especially chemiluminescent substrates for future, and extend the use of this approach to detection of pathogenic viruses in blood serum.

**Chapter 3: TOWARDS THE ISOLATION AND CHARACTERIZATION OF
SINGLE-STRANDED DNA APTAMERS SPECIFIC TO THE MURINE IgG
HYHEL-5**

3.1 Introduction

Aptamers are short, functional single-stranded nucleic acid molecules (single stranded-DNA and RNA) isolated from large random libraries through a process termed *Systematic Evolution of Ligands by EXponential enrichment*, or SELEX (75), (74). Aptamers have a specific and complex three-dimensional shape characterized by stems, loops, hairpins, bulges, pseudoknots, and quadruplexes. These three-dimensional structures allow them to bind to a wide variety of targets from single molecules to complex target mixtures (76) (Figure 3-1). This binding occurs due to structure complementarity, stacking of aromatic rings, hydrogen bonding, electrostatic and *van der Waals* interactions or a combination of these effects (78).

SELEX consists of multiple rounds of binding and amplification of the isolated binding population that are used to narrow the library down to only the highest affinity nucleic acids. In contrast to many other biomedical methods, absolutely no information about the target molecule is necessary.

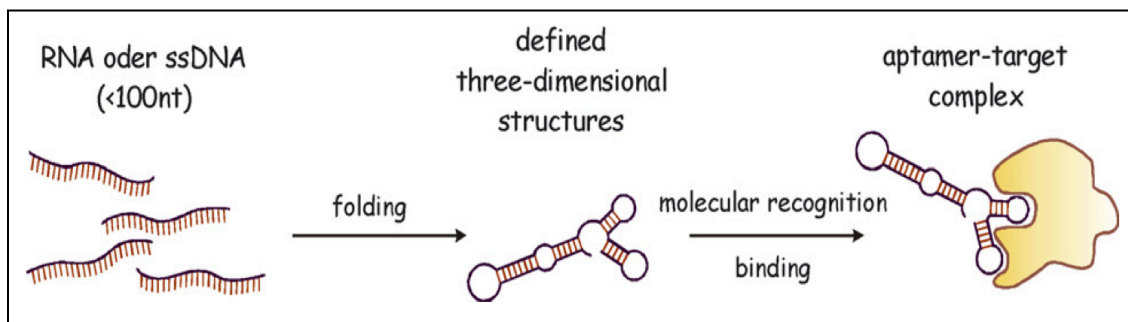


Figure 3-1: Schematic representation of the functionality of aptamers (Adapted from (76))

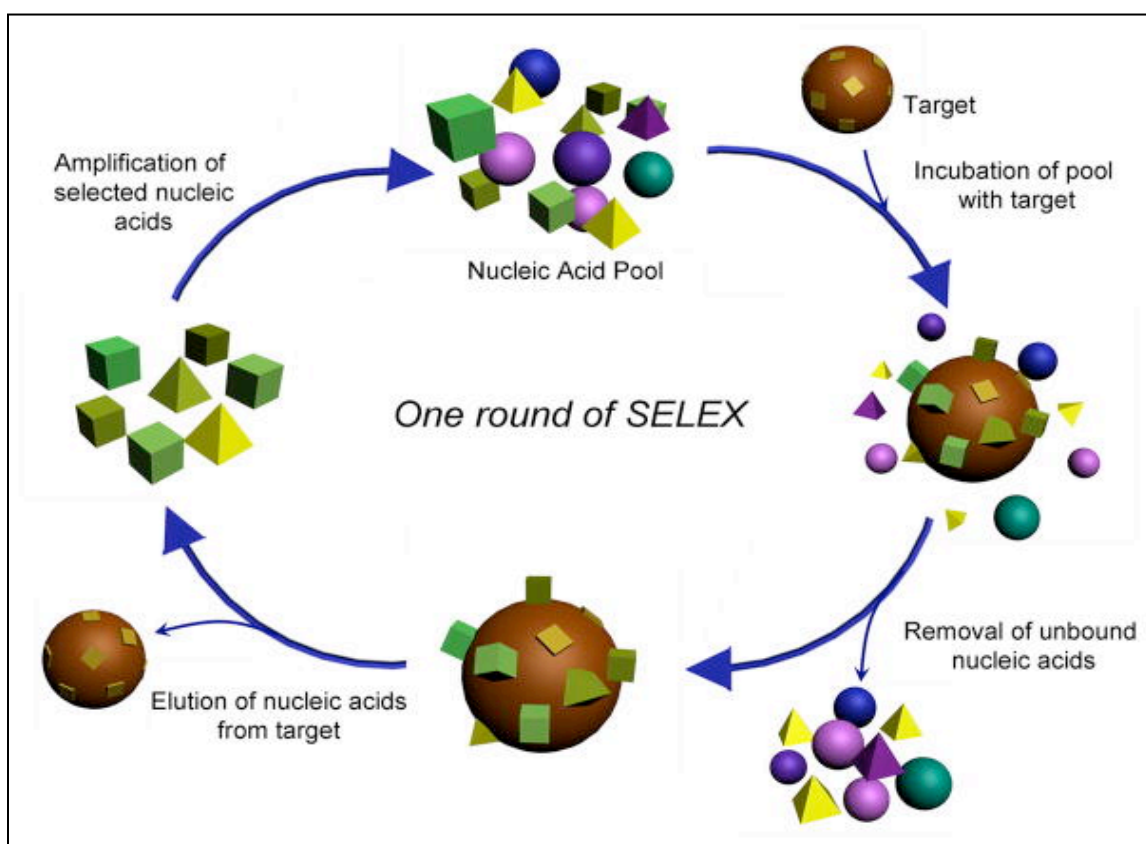


Figure 3-2: In vitro selection of target specific aptamers using SELEX

The procedure starts with a synthetic random DNA oligonucleotide library that consists of multitudes of single stranded DNA fragments ($\sim 10^{13}$ - 10^{15}) with unique sequences. This library is then incubated with a target molecule. Unbound nucleic acids are removed and the bound nucleic acids are eluted from the target molecule. The new pool is regenerated from those that bound to the target after amplification. The process is repeated multiple times to obtain high affinity binders (Figure adapted from (125)).

Aptamers have recently attracted increased attention for their potential application in many areas of biology and biotechnology. They are used in sensors (126,127) or to regulate cellular processes (128), as well as to guide drugs to their

specific cellular target (129). Although aptamers are analogous to antibodies in their adaptability and range of application, they display several potential advantages over their protein counterparts. They are smaller, faster and more economical to produce, may show improved affinity and specificity, are highly biocompatible and non-immunogenic and can easily be modified chemically to yield improved properties (76). The specificity and characteristics of the aptamers are determined by their tertiary structure instead of primary sequence (130). They do not require animals for their production in contrast to conventional methods of making antibodies. Aptamers can also have longer shelf life since they are more resistant to denaturation and aggregation (76,131,132). For clinical applications, the fact that aptamers can be prepared fully *in vitro* may allow for a faster regulatory approval process (133). In fact, Pegaptanib, a modified RNA aptamer targeting vascular endothelial growth factor, a protein that induces pathological angiogenesis and stimulates vascularization, was in 2006, the first FDA approved aptamer based drug (134). It is currently being used successfully and safely to treat age related macular degeneration (135).

Antibodies constitute molecular recognition elements for a wide range of applications. They can recognize their antigens with great specificity and therefore are powerful tools in basic research. This property of the antibodies has allowed researchers to identify molecules and by manipulating these molecules can study the function of proteins of interest. They provide valuable tools in the fields of diagnostics and have proven valuable in several diagnostic assays (132). While the

discovery of monoclonal antibody technology has revolutionized immunology research and further broadened the applications of antibodies, they exhibit certain limitations such as sensitivity towards temperature, irreversible denaturation, and requirement of animal sources for their production that may lead to batch-to-batch variation. Their high molecular weight makes them difficult for site-specific labeling.

Various approaches have been developed to circumvent these limitations such displaying peptide libraries on phages (136,137) and ribosomes (138,139), antibody engineering (140) etc. As an alternative, aptamers, an entirely different class of ligands, can address the short-comings of antibodies. The development of the SELEX technology has made possible the screening of aptamer ligands that are able to recognize virtually any class of target molecules with high affinity and specificity. These oligonucleotide sequences or aptamers rival antibodies in both therapeutic and diagnostic applications. They mimic properties of antibodies in a variety of diagnostic formats. The demand for diagnostic assays in the management of existing and emerging diseases has increased over the years, and aptamers are suitable for applications based on molecular recognition needs in these assays. As compared to expensive antibodies, aptamer based diagnostic assays would provide cost-effective tools in the diagnosis (132).

In this chapter, we describe the screening of DNA aptamers against the monoclonal IgG HYHEL-5 antibody. HyHEL-5 is a murine IgG1 κ antibody that is specific for the antigen Hen Egg Lysozyme (HEL), the most studied protein antigen (141). HyHEL-5 is required for various research purposes such as studying protein-

protein interactions, mapping antigenic sites etc. We attempted to isolate DNA aptamers against HyHEL-5. Anti-IgG aptamers could be used as model systems in various applications, such as studying protein-DNA interactions, aptamer-based diagnostic assays where it can be used as a recognition element or as labels in immunoassays.

3.2 Materials and Methods

3.2.1 SELEX procedure (*In vitro* selection)

For the selection of aptamers, a combinatorial library of 82-mer ssDNA oligonucleotides of sequence 5'-TCCAACCTTCATCAGCCACC-N40-GGTAAGAGAGGGAGGAATGG-3' was used. Each oligo in this library consisted of a randomized 40 nucleotide core sequence (N40), flanked by two conserved primer binding sites of 20 and 22 bases, respectively. The conserved primer binding sites aid in amplifying the core sequences during the SELEX process. Theoretically such a library would contain 4^{40} unique random sequences. But generally in practice, the libraries contain 10^{14} to 10^{15} different species. A phosphate group was introduced into the 5' end of the reverse primer. Following PCR amplification, the dsDNA obtained by amplification was treated with phage lambda exonuclease, which cleaves the phosphorylated strand of DNA prior to the next round of SELEX (142). Based on published procedures for SELEX protocols, 10 rounds of SELEX were conducted before sequencing the enriched aptamer population. The experiment

consisted of three parts: (1) Initial amplification; (2) Binding of aptamers to the target; (3) Cloning of the enriched single-stranded oligos.

3.2.1.1 Initial Amplification

For the initial amplification of the random library, 500 pmol of the ssDNA oligo were combined with 300 pmol of each primer and 50 μ L of the Promega GoTaq PCR master-mix (Promega Corporation, Madison, WI, USA) in a total reaction volume of 100 μ L. The PCR cycles consisted of the following conditions: 2 min at 95 $^{\circ}$ C, followed by 20 cycles of 10 s at 95 $^{\circ}$ C, 10 s at 65 $^{\circ}$ C and 10 s at 72 $^{\circ}$ C, followed by 5 min extension at 72 $^{\circ}$ C (product formation of a random library reaches its maximum after 20 cycles (143)). After the PCR reaction, dsDNA was treated with lambda exonuclease, which selectively digests the phosphorylated strand of dsDNA. Thus the single strand DNA obtained was used for binding experiments with the target.

3.2.1.2 Binding of the ssDNA to the target

Nitrocellulose membrane filters (0.45 μ m pore size, Millipore Corp) were used to separate the DNA-antibody complexes from the unbound ssDNA pool. The ssDNA pool was denatured at 95 $^{\circ}$ C for 10 min and then cooled to room temperature (25 $^{\circ}$ C) over 10 min. Before the incubation of the ssDNA pool with the protein target, a negative selection was performed to remove any filter-binding species that may be in the DNA population. It was done by filtration of the ssDNA pool in the absence of target. The flow-through containing the unbound ssDNA fraction was used for the

binding reaction and selection procedure. Murine anti-HEL monoclonal HyHEL-5 antibodies were produced from hybridoma cells and purified by Protein G Affinity Chromatography by Biovest/National Cell Culture Center (Minneapolis, MN). The ssDNA pool was incubated with the HyHEL-5 antibody at room temperature for 1 hr in binding buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 2 mM DTT, and 1% BSA). The IgG-DNA complex was then filtered through a 0.45 µm HAWP nitrocellulose acetate filter (Millipore). The filter was washed with 1 ml binding buffer. In order to increase stringency throughout the SELEX process, the number of washes was gradually increased. The ssDNA retained on the filter was eluted at 95 °C with 7 M urea for 5 min and subsequently recovered by ethanol-precipitation.

3.2.1.3 DNA cloning and sequencing

After 10 rounds of SELEX, the enriched ssDNA pool was amplified one more time and the PCR products were cloned using the pGEM®-T Easy vector systems. The vector supplied in the kit has a 3' terminal thymidine (Promega, USA). These single 3'-T overhangs at the insertion site improve the efficiency of ligation of a PCR product into the plasmids by preventing re-circularization of the vector and providing a compatible overhang for PCR products generated by Taq polymerases. PCR products of the 10th round were purified by QIAquick PCR purification kit (Qiagen) that removed any unincorporated nucleotides, primers and polymerase. The cloned inserts from 48 individual plasmids were sequenced by SeqWright Genomic services (SeqWright, Houston, TX, USA). The sequenced results were

analyzed by Clustal-Omega [<http://www.ebi.ac.uk/Tools/msa/clustalo/>], a multiple sequence alignment program. The aptamer cluster that revealed conserved areas or consensus motifs were grouped together and their secondary structures were predicted by Mfold web server (144). Individual aptamers best representing possible consensus sequences were synthesized and tested by ELONA (Enzyme Linked Oligonucleotide Assay) and Surface Plasmon Resonance (SPR) for binding experiments with HyHEL-5 antibody and characterized.

3.2.2 Primary and secondary structures of the aptamers

The sequences were aligned using Clustal-W web program. Secondary structures of the selected aptamers were predicted by the Zuker algorithm using Mfold (mfold.rna.albany.edu).

3.2.3 Characterization of the selected and enriched DNA aptamers

A few (8-10) aptamers best representing possible consensus sequences of the sequenced oligos and secondary structures were chosen for further analysis and were synthesized by IDT. High affinity DNA aptamers generated by the repeated *in vitro* selection procedure were expected to bind to the variable region of HyHEL-5 monoclonal antibody. To analyze the binding affinity of the HyHEL-5 (IgG) aptamer to the antibody, enzyme linked oligonucleotide assay (ELONA) and SPR experiments were employed.

3.2.3.1 Enzyme Linked Oligonucleotide Assay to investigate the binding affinity of the selected aptamer to HyHEL-5

An Enzyme Linked Oligonucleotide Assay was carried out to assess the binding of the aptamer to the target. ELONAs can detect the presence of analytes (by using aptamers as capture molecules) or detect the presence of aptamers (by using the corresponding analyte as the capture molecule). DNA aptamers with a biotin label at the 5' end were synthesized by IDT. A 96 well microtiter plate was coated overnight at 4 °C with 100 µL 5 µg/mL HyHEL-5 antibody in 50 mM carbonate buffer (pH 9.6). The wells were subsequently washed three times with binding buffer (section 3.2.1.2) supplemented with 0.05% Tween-20 and then blocked with 100 µL binding buffer containing 1% (w/v) bovine serum albumin (BSA) for 1 hr at 37 °C. The wells were then washed three times with 200 µL binding buffer containing 0.05% Tween 20. A concentration series of 50 µL aptamer solution ranging from 1 nM to 1000 nM in binding buffer were added to each well and the mixture incubated for 1 hr at 37 °C. A well with no aptamer served as the negative control. DNA from the random library was also used as a second negative control. The plate was again washed and 50 µL Extravidin-HRP (Sigma) was added to the plate and incubated for 1 hr at 37 °C. The wells were then washed further three times and color developed by adding 100 µL of 1 step ultra TMB ELISA substrate and kept for 30 min at 25 °C. The reaction was stopped with 50 µL 1 M H₂SO₄ and absorbance measured at 450 nm in a Tecan Infinite M200Pro microplate reader.

3.2.3.2 Surface Plasmon Resonance (SPR) binding studies

To quantify the binding affinity of the selected DNA aptamer to HyHEL-5 in greater detail, SPR was investigated. SPR detects changes in the refractive index in the immediate vicinity of the surface layer of a gold sensor chip. SPR is observed as a sharp shadow in the reflected light from the surface at an angle that is dependent on the mass of material at the surface. There is a shift in the SPR angle when biomolecules binds to the surface and change the mass at the surface. This change in the resonance angle can be monitored in real time as a plot of resonance signal versus time (Figure 3-3).

The real-time measurement of the interaction between the selected aptamers and HyHEL-5 was performed by SPR using BIAcore 2000 (GE healthcare). For this experiment a streptavidin sensor was purchased from GE Healthcare. Sensor Chip SA is designed to bind biotinylated molecules. It allows for immobilization of nucleic acids through a 5'-biotin moiety. Biotinylated T16 oligomers were employed that could be immobilized on the streptavidin surface. The aptamers were synthesized with 3'polyA tail of 16-mers that were able to hybridize with the T16 oligomer on the SA chip temporarily. The bound aptamer on T16 oligomer was removed by passing 6 M urea and then SA chip was reused for hybridization for other aptamers via T16 and polyA tail complementary pairing. Biotinylated T16 oligomers and aptamers that revealed binding to HyHEL-5 in the ELONA experiment were synthesized from IDT with a 3'polyA tail of 16-mers. As a positive control, a known RNA aptamer to human IgG was also synthesized by IDT. The SPR assays were

performed as described by Miyakawa *et al.* (145). The experimental strategy is shown in Figure 3-4. Biotinylated T16 oligos were first bound to the sensor chip surface of flow cell 1 and 2. Then the aptamers with their polyA tail were immobilized in flow cell 2 by complementary hybridization to the dT16 oligomer. HyHEL-5 solution (of different concentration between 25 nM and 100 nM) was injected through the flow cells 1 and 2 of the sensor chip. Data were analyzed by subtracting the flow cell 1 data from the flow cell 2 data that show a net interaction between aptamer and protein. The sensor chip was regenerated by removing the bound aptamer by injecting 6 M urea. The resulting sensorgram was analyzed using Scrubber 2.0 (University of Utah, UT, USA) software.

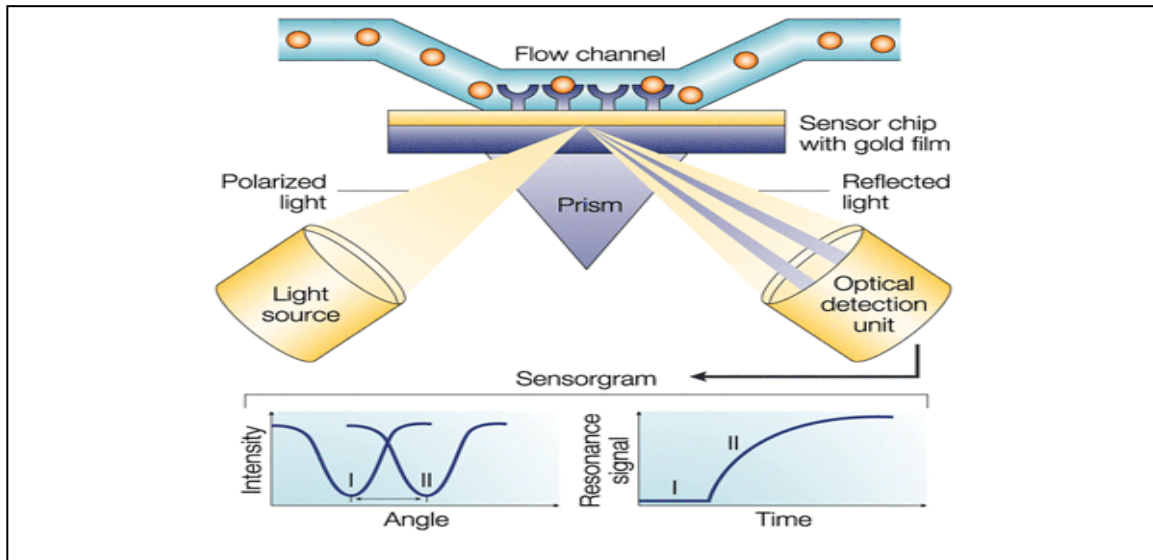


Figure 3-3: Schematic of Surface Plasmon Resonance

SPR detects changes in the refractive index in the vicinity of the surface layer of a sensor chip. Binding of biomolecules shifts the SPR angle, which can be monitored in real time as a function of the resonance signal versus time (adapted from (146)).

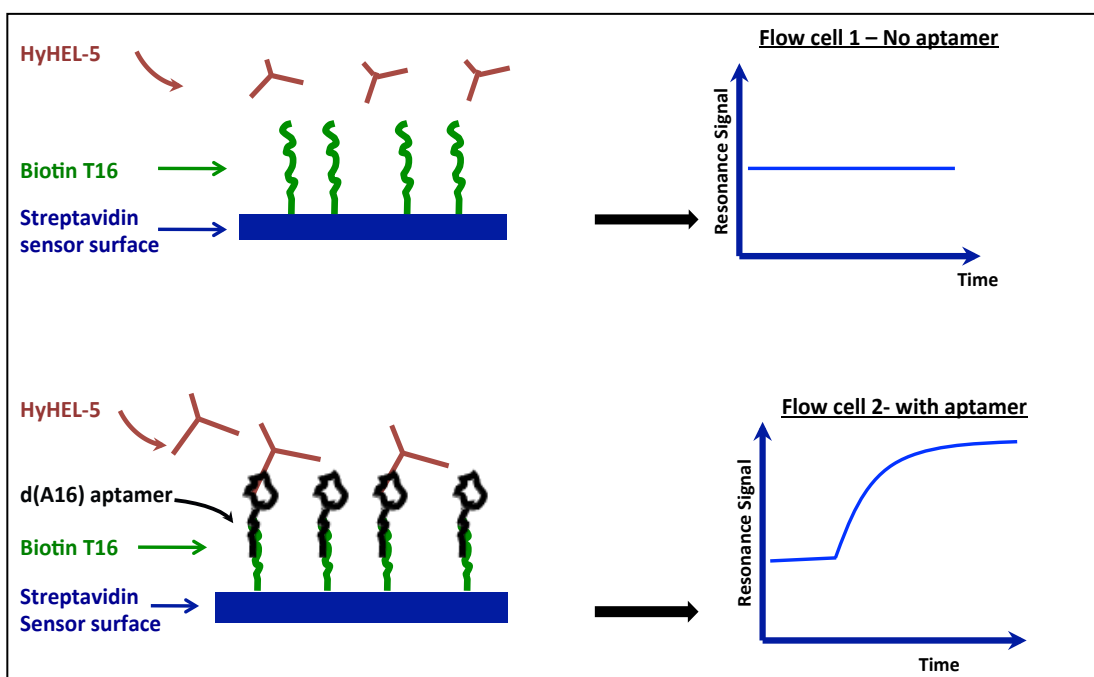


Figure 3-4: Experimental strategy for aptamer characterization using SPR

Biotinylated T16 oligo are bound to the sensor chip surface of flow cell 1 and 2 via avidin-biotin linkage. Aptamers with polyA tail are immobilized in flow cell 2 by complementary hybridization to the dT16 oligomer. HyHEL-5 (of different concentration between 25 nM and 100 nM) is injected through the flow cells 1 and 2 of the sensor chip.

3.3 Results and Discussions

3.3.1 *In vitro* selection

Initial amplification of the single-stranded DNA library: For the selection of HyHEL-5 aptamers, ssDNA library with a random sequence of 40 nucleotide was used as precursor pool. The random region was flanked by primer binding sites for the PCR amplification. The reverse primer was synthesized with a 5' terminal

phosphate for ssDNA generation from dsDNA PCR product by lambda exonuclease digestion (Figure 3-5).

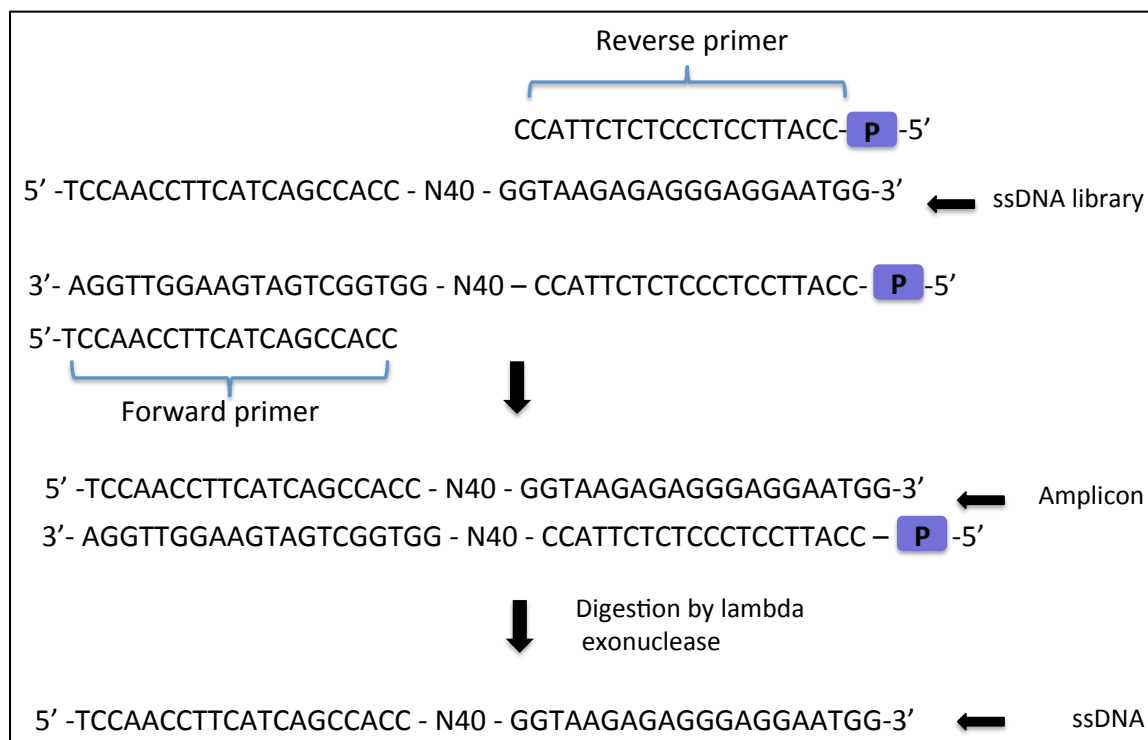


Figure 3-5: Design of the ssDNA library for HyHEL-5 specific aptamer selection

For the first round of selection, 500 pmol of the ssDNA library was used directly for the PCR amplification. After the PCR, a small sample volume of the DNA was analyzed by agarose gel electrophoresis on a 2% agarose gel stained with Sybr Gold nucleic gel stain (Life Technologies, Grand Island, NY, USA). Initial amplification of the single stranded DNA library yielded an 82 bp fragment on the gel (Figure 3-6).

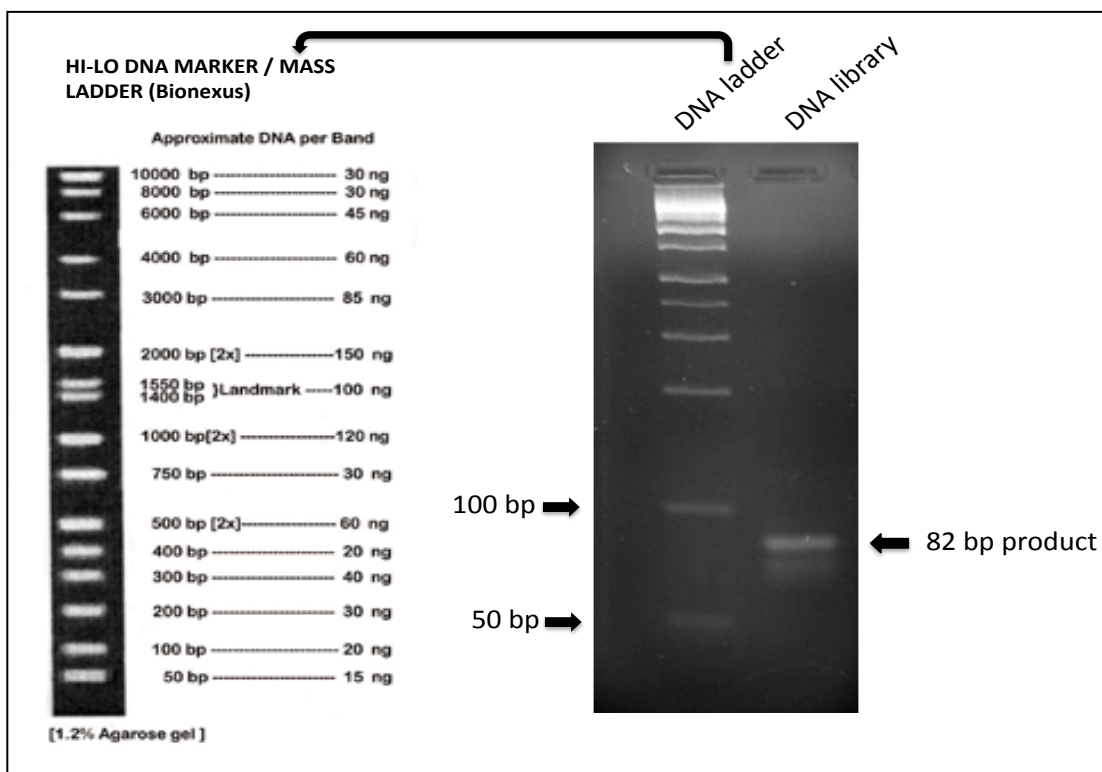


Figure 3-6: PCR product from initial amplification of the ssDNA library

Performing cycle-course PCR: One of the most important aspects in PCR is not to over-amplify the selected templates, since amplification artifacts can dominate a selection (143). To determine the optimal number of cycles for amplification, a cycle-course PCR was performed. A small sample of DNA was taken from each PCR cycle, and saved for gel analysis. The cycle at which a single strong band is present was the cycle that was used to amplify the remainder of the pool (as shown in Figure 3-7). Here we selected 10 cycles with 0.25 ng of template for the rest of the SELEX cycles.

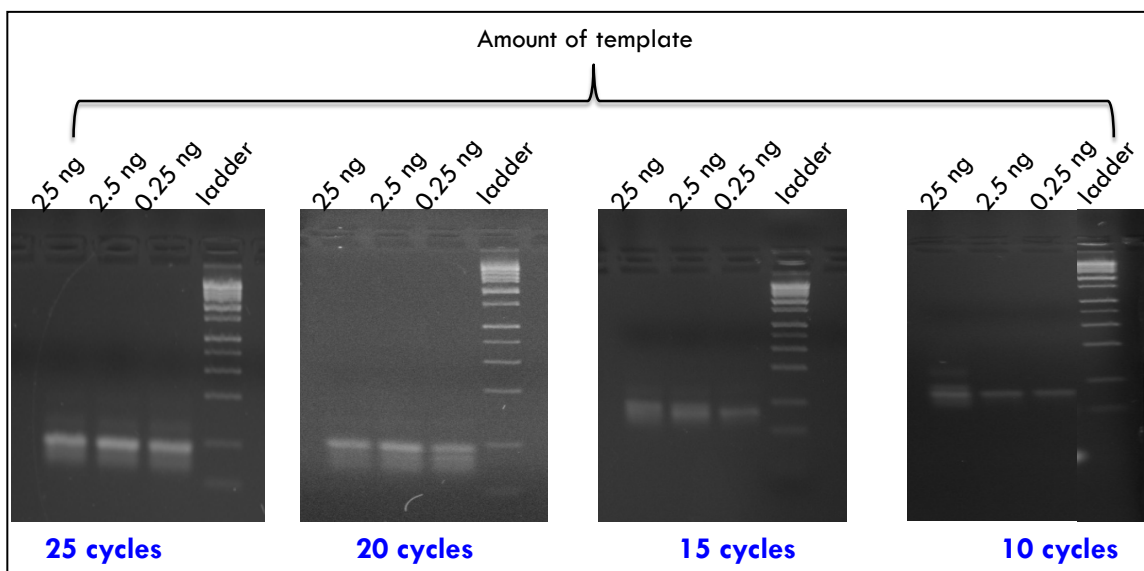


Figure 3-7: Optimizing the PCR cycles SPR

PCR optimization was done for the amount of target used and number of cycles. Different amounts of target (25 ng, 2.5 ng and 0.25 ng) were subjected to various number of PCR cycles (10-25) to see at what concentration and cycle we get a specific single band.

After PCR amplification, the double-stranded product was treated with lambda exonuclease, a 5' to 3' nuclease that specifically attacks dsDNA on a 5' terminal phosphate (147). Therefore, the strand of DNA synthesized from the phosphorylated primer was digested by the exonuclease leaving the complementary strand for the next round of selection.

For the exonuclease digestion, 1 µg of purified ds DNA was treated with 25 U lambda exonuclease in 200 µL reaction according to the manufacturer's instructions (New England Biolabs (NEB), USA). It was important to remove any lambda

exonuclease from the ssDNA pool for the following cycles. Therefore, after lambda exonuclease digestion, phenol/chloroform extraction and subsequent ethanol precipitation were performed to eliminate the lambda exonuclease from the aptamer pool. The ssDNA pool so obtained was used for subsequent SELEX cycles.

3.3.2 Primary and Secondary Structures of the Aptamers

Sequence analysis

The ssDNA obtained from round 10 was amplified one more time and PCR products were cloned using the pGEM®-T Easy vector system. The cloned inserts from 48 individual plasmids were sequenced by IDT. The sequenced results were analyzed by Clustal-W web program. Based on the sequencing analysis, seven sequences were represented more than once in the clones. Aptamer 2E was represented most frequently (nine times). The other fourteen variants were different orphan sequences (Table 3-1). Alignment of these sequences revealed some conserved areas of (GGGT) in 11 different ligands (Figure 3-9).

CLUSTAL -W multiple sequence alignment

```

1E      GCCACCTGAAACGATTGCACGGCCCTTGGGTTGCTTGAGT----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGA----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAATGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGTGGGGGG----- 40
2E      -----TGAAACGATTGCACGGCCCTTGGGTTGCTTGAGCGGGGGG----- 40
3E      -----GGAAACGATCGCACGGCCCTTGG-TTTCAGGANTGGGGGG----- 39
4E      -----GTGGACATTGTTTCGTAGGAGTGGTTTGTCTGGGTGGCAGC----- 40
4E      -----GTGGACATTGTTTCGTAGGAGTGGTTTGTCTGGGTGGCAGC----- 40
5E      -----CGGGACCTCGCTTGGGATGCGGGCACCGTTGGGTAAAGGC----- 40
6E      -----TCGATTGGTATGTAATAGCTTTGCGCGAGCGGGCGGGGG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAGGACGCGTG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAGGACGCGTG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAGGACGCGTG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAGGACGCGTG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAGGACGCGTG----- 40
7E      -----GATGTGTAGCATTGACGCTTCGTATCGGAGAATAGGCGTG----- 40
8E      -----TTCTTCTGTTTTAACGTAATAGCTACCTGGCTACGGGGTG----- 40
9E      -----TGACCTTTGTGCTGTGTGTGCTAGCGTATTAGCTACGGGG----- 40
10E      -----CCTATCCATTTGTTGCTTGGGATGCACGCGGATGGGCGCT--- 40
10E      -----CCTATCCATTTGTTGCTTGGGATGCACGCGGATGGGCGCT--- 40
11E      -----TTTCTGTGCGCGGGTTGAGCTATTGGTTCTCCATTAGGGT-40
12E      -----TTGAACATACTGTG-ACGCGCTGAGCTGT-GGCCGGCCG--AGG-- 40
13E      -----TGCAGTGTTTATTGTGCCGGGCTAGTTATTGTGACCCGGT----- 40
13E      -----TGCAGTGTTTATTGTGCCGGGCCAGTTATTGTGACCCGGT----- 40
14E      -----GGTTCTGTCTAGGGGTTGGGGCGGTGGCGGCGCGCTAGTC-40
15E      -----TACACCTGGTTCGTAGATGTTTCGAAA-CTTCGTCATGCAGCT-----40
16E      -----CGAGTGTTTCGTTTGGCCACGCAG-CTGTAG-ATAGAGCCCAT-- 40
17E      -----TGCCGGGTGCTTGCATCTGAACTCTACGTGACGC-GCGTGT-- 40
17E      -----TGCCGGGTGCTTGCATCTGAACTCTACGTGACGC-GCGTGT-- 40
17E      -----TGCCGGGTGCGCGCATCTGAACTCTACGTGACGC-GGGGGA-- 40
18E      -----CGATTGCCGAGATTTGTATTGAGCAGGGTGCAGCGCATCC 40
18E      -----CGATTGCCGAGAGTTGTATTGAGCAGGGTGCAGCGCATCC 40
19E      -----GGGGGGTATATAGTCAGCGTCTGGTTCCGAGTTGAGGGTG----- 40
20E      -----GAGCGTGCTACTTTGAAACCTCATGTGCGTGTTTCGTCTCG----- 40

```

Figure 3-8: The sequencing results of selected clones were analyzed by multiple sequence aligning via Clustal-W that revealed their frequencies (Table 3-1).

Table 3-1: Frequency of sequences of the selected clones

Aptamer	Number of clones	Nucleotide length
1E	1	40
2E	9	40
3E	1	40
4E	2	40
5E	1	40
6E	1	40
7E	7	40
8E	1	40
9E	1	40
10E	2	40
11E	1	40
12E	1	40
13E	2	40
14E	1	40
15E	1	40
16E	1	40
17E	3	40
18E	2	40
19E	1	40
20E	1	40

```

1E  ----GCCACCTGAAACGATTGCACGGCCCTT---GGGT---TGCTTGAGT-----40
2E  ----TGAAACGATTGCACGGCCCTT-----GGGTTGCTTGAGTGGGGGA---40
3E  ----GGAAACGATCGCACGGCCCTTGTTTTCAGGANT-GGGT-GGG-----40
4E  ----GTGGACATTGTTTCGTAGGAGTGGTTTGTC---GGGT---GGCAGC-----40
5E  ----CGGGACCTCGCTTGGGATGCGGGCACCGTT---GGGT---AAAGGC-----40
8E  ----TTCTTCTGTTTTAACGTAATAGCTACCTGGCTACG-GGGT-G-----40
11E ----TTTCTGTGCGCGGGTTGAGCTATTGGTTCTCCATTAGGGT-----40
14E ----GGTTCTGTCTAG-----GGGTGGGGCGGTGGCGGCGCGCTAGTC---40
17E ----TGCC-----GGGTGCTTGCATCTGAACTCTACGTGACGCGCGTGT---40
18E ----CGATTGCCGAGATTTGTATTGAGCA-----GGGTGCAGCGCATCC-----40
19E ----GGGGGGTATATAGTCAGCGTCTGGTTCCGAGTTGA--GGGT-G-----40

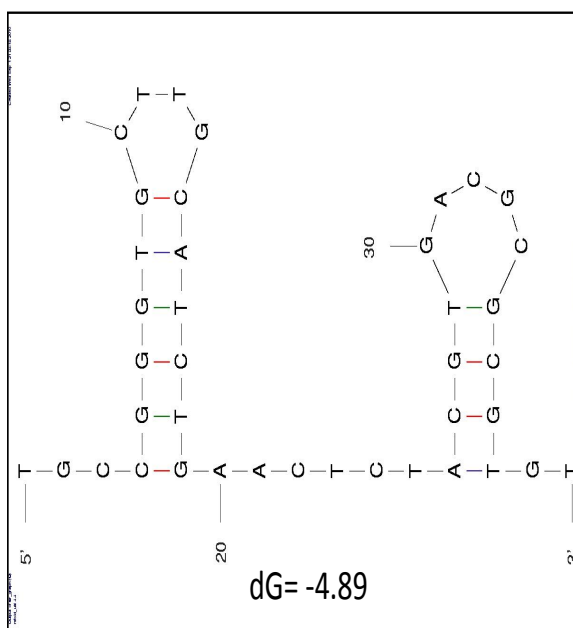
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Figure 3-9: Sequences containing the consensus motif GGGT

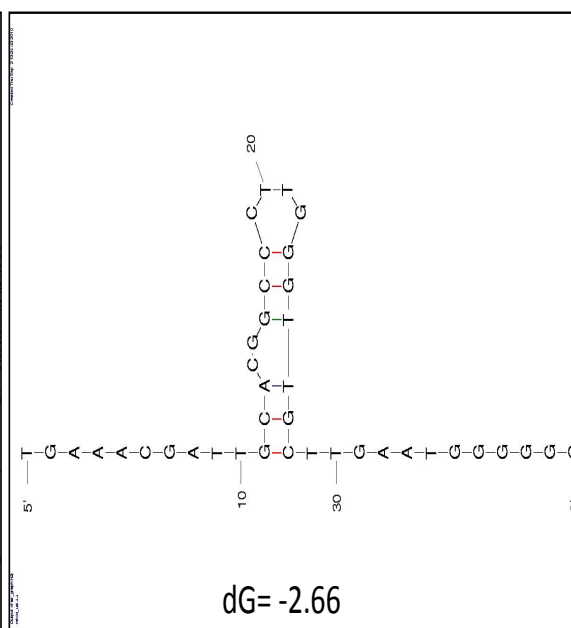
Secondary Structure

Secondary structures of the selected aptamers were predicted by Mfold. Individual aptamers best representing possible consensus sequences of the sequenced oligos were synthesized and tested for binding experiments with HyHEL-5 antibody and characterized.

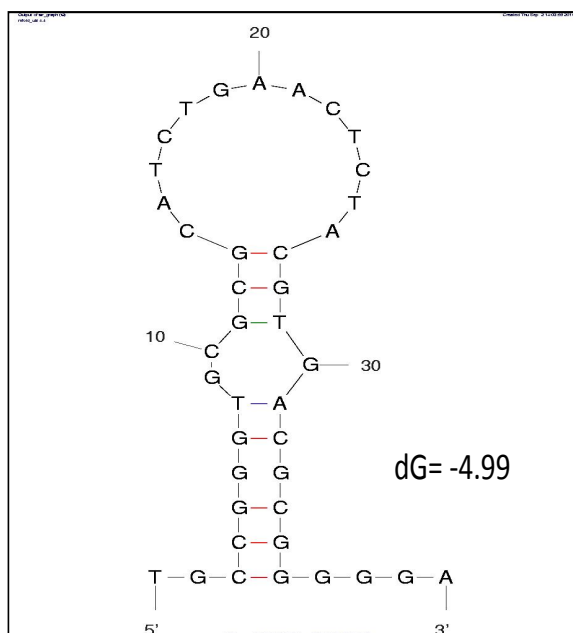
According to the proposed secondary structures, the eleven variants (1E, 2E, 3E, 4E, 5E, 8E, 11E, 14E, 17E, 18E, and 19E) were folded into different structures. There were no common secondary structure elements that could be assigned to these variants. We selected four aptamers that exhibited some interesting secondary structures (such as hair-pin or loop) to test for binding. They represent the commonly occurring structural motifs from all the aptamers. Their secondary structures are shown in Figure 3-10).



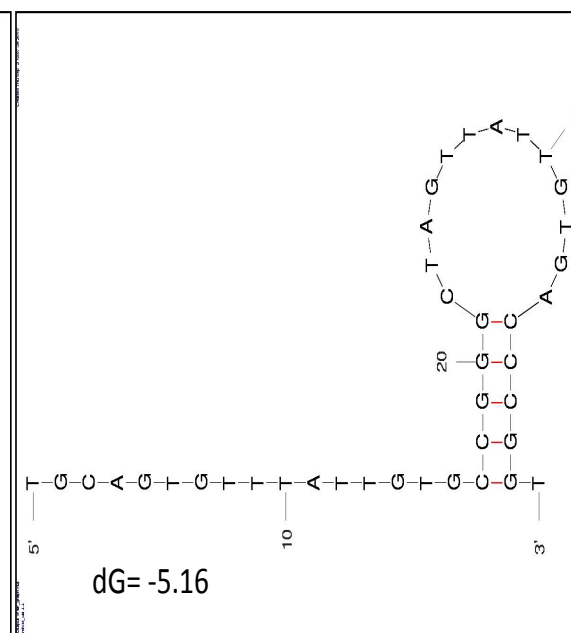
Aptamer 2E



Aptamer 8E



Aptamer 18E



Aptamer 11E

Figure 3-10: Mfold secondary structure of aptamers (2E, 8E, 18E, 11E)

3.3.3 Characterization of the selected and enriched DNA aptamers

A few aptamers (2E, 8E, 11E, 18E) best representing possible consensus sequences of the sequenced oligos and secondary structures were chosen for further analysis and were synthesized from IDT.

3.3.3.1 ELONA

Four putative aptamers (2E, 8E, 11E, 18E) for HyHEL-5 were tested for binding to HyHEL-5 by ELONA as described above in Section 3.2.3.1. These aptamers were synthesized with a 5' biotin modification. HyHEL-5 was used as capture antibody for the biotinylated aptamers. The results of all four aptamers are shown in Figure 3-11. We did not see a significant increase in absorbance values with increasing concentration of the aptamer, indicating that the aptamers did not bind to HyHEL-5. The absorbance values of all the concentration of the aptamers were almost similar to background values with no aptamer control. However aptamer 18E showed a slightly higher absorbance as compared to the other aptamers (2E, 8E and 11E). Therefore, we further carried out real-time SPR analysis of aptamer 18E to explore if there is any kind of binding affinity of 18E towards HyHEL-5. SPR binding studies were also performed with 2E, 8E and 11E aptamers.

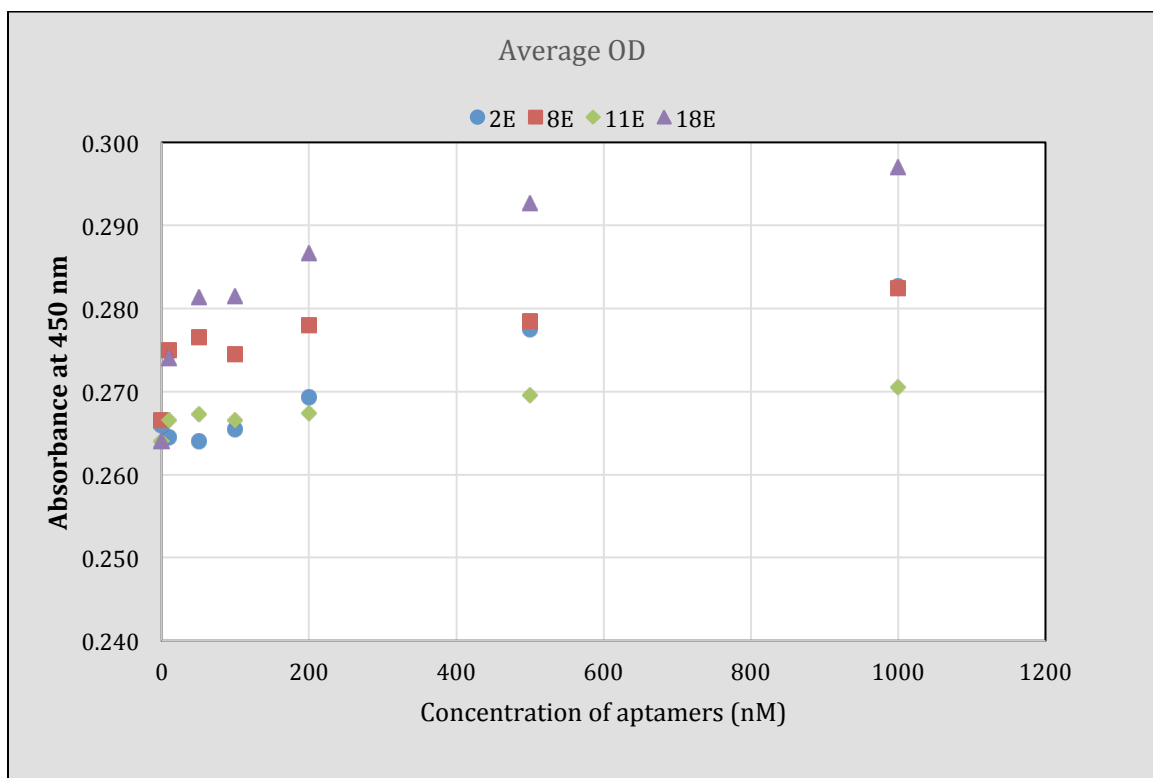


Figure 3-11: Detection of binding of aptamers 2E, 8E, 11E and 18E in ELONA

The range of the assay was from 1 nM to 1000 nM.

3.3.3.2 Surface Plasmon Resonance

The real-time SPR measurements of the interaction between the selected aptamers and HyHEL-5 were performed on a BIACore 2000 instrument. For the SPR experiments a streptavidin sensor chip was employed. As a positive control a known RNA aptamer to human IgG (145) was also analyzed. This RNA aptamer (Apt 8-2) was isolated by Miyakawa *et al.* and shown to bind to the Fc portion of human IgG. It is a 23-mer aptamer (ggAGGUGcUccgaaaGGaaCUcc) highly specific to human IgG, but not to any other species IgGs (Figure 3-12).

showing the net interaction between aptamer and protein. The sensor chip was regenerated by removing the bound aptamer by injecting 6 M urea. The binding of the RNA aptamer to human IgG is depicted in the Figures 3-13, 3-14, and 3-15. Figure 3-13 shows an increase in the response units upon immobilization of 5' biotinylated oligo dT16 on the surface of chip in both Flow cell 1 and Flow cell 2 thus confirming the successful immobilization of the dT16 oligomer on the streptavidin surface in both the flow cells. After the immobilization of the oligo dT16 on the chip surface, RNA aptamer with polyA tail was offered in flow cell 2 but not in flow cell 1 (Figure 3-14); the increase in the response units as the aptamer is passed indicates the successful hybridization of the oligo dT16 to the complementary polyA tail of the aptamer. Finally human IgG was passed on the flow cells (Figure 3-15). There is an increase in the response over time that reveals that the RNA aptamer binds to the human IgG. This confirms that the positive control RNA aptamer binds to human IgG and hence the proper functioning of our assay. The sensor chip was then regenerated by removing the bound RNA aptamer by passing 6 M urea. The HyHEL-5 aptamers were then bound on the oligo dT16 via polyA tail complementary hybridization and tested for binding to HyHEL-5 in the similar way as the RNA aptamer for IgG binding. The HyHEL-5 aptamers did not show binding to HyHEL-5 antibody in the SPR measurements (data not shown).

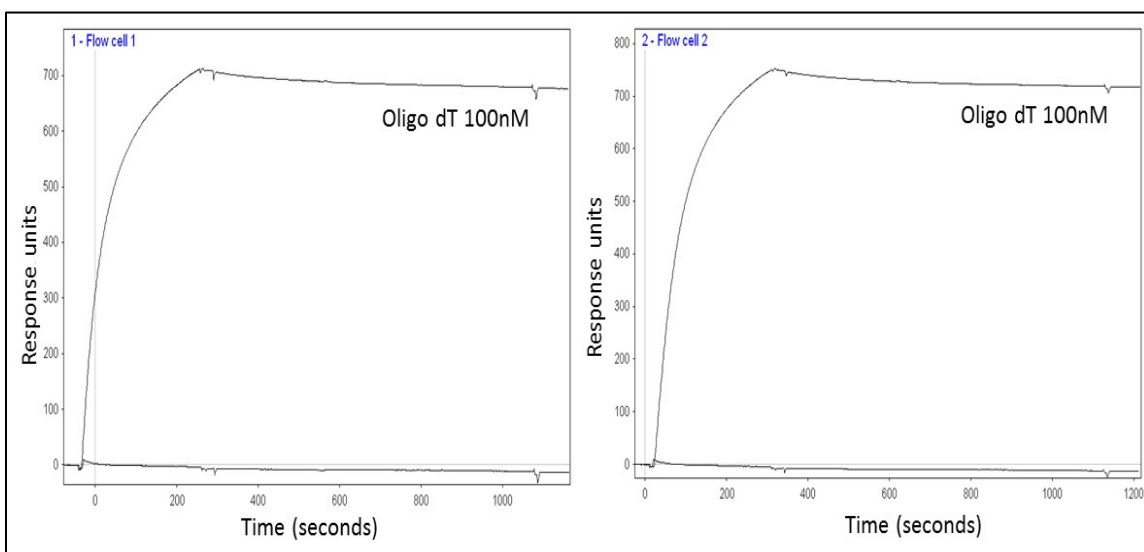


Figure 3-13: Immobilization of 5' biotinylated oligo dT16 on the surface of streptavidin chip in Flow cell 1 and Flow cell 2

100 nM of oligo dT16 was passed in both the flow cells. The sensorgram was obtained using Scrubber 2.0 software.

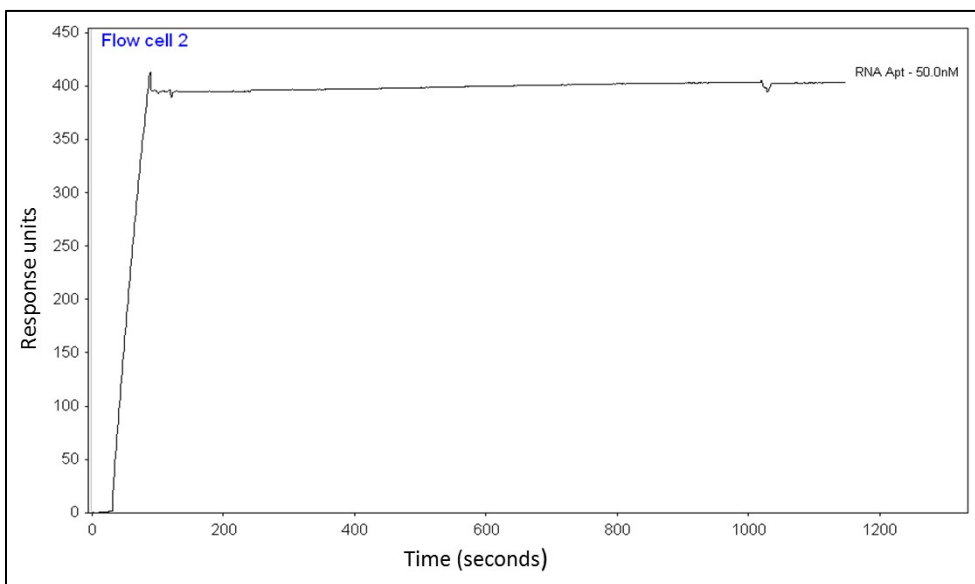


Figure 3-14: 50 nM RNA aptamer passed in flow cell 2 but not in flow cell 1

The resulting sensorgram was analyzed by scrubber 2.0.

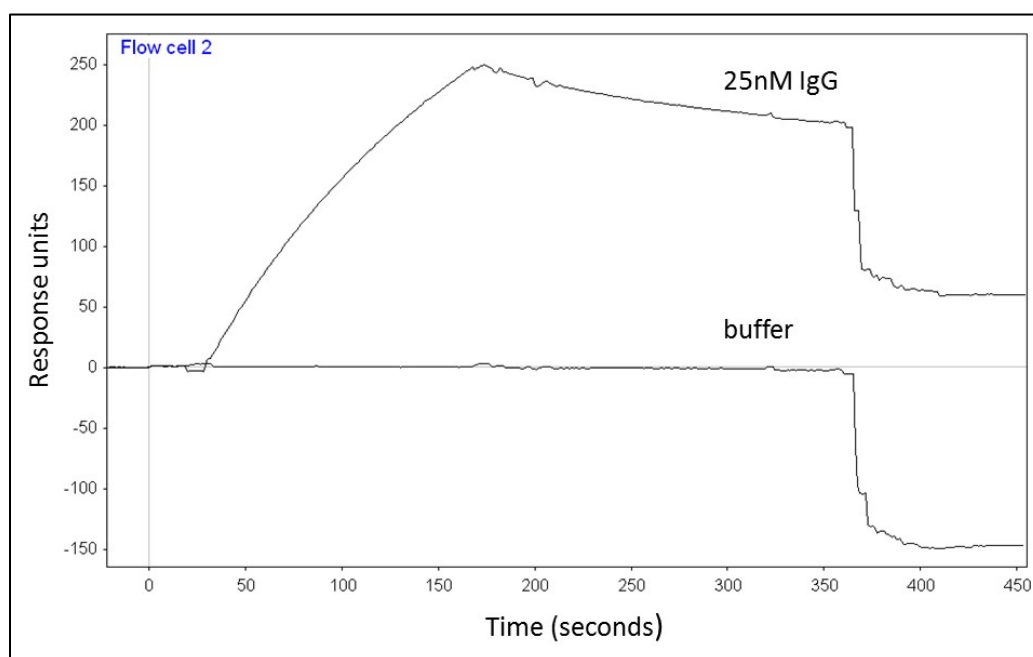


Figure 3-15: 25 nM of IgG was passed in both the flow cell 1 and flow cell 2

For regeneration 100 mM phosphoric acid was used. The response units were plotted over time using Scrubber 2.0 software.

The success of SELEX process for screening high affinity ligands depends on various factors such as temperature, salt concentrations, pH, and washing. Sometimes, functionally less potent molecules, may be overrepresented due to the fact that they have the ability to replicate slightly better during PCR than their competitors. Moreover, the SELEX process for the selection of target-specific aptamers is a universal process consisting of few repetitive steps (binding, partition, elution, and amplification) and there is no standardized aptamer selection protocol for any target. The SELEX design and the selection conditions depend on the target itself,

the oligonucleotide library or the desired features of aptamers and their applications. For future, we plan to improve our SELEX conditions by increasing the stringency, include counter-selection steps in each cycle that may help in getting successful binders. The goal of our aptamer screening was to identify high affinity DNA aptamers specific to HyHEL-5 (a model IgG) that can be utilized as a bio-recognition element. Since the aptamer screening did not yield successful binding ligands, we focused on a well-known aptamer, i.e., IgE aptamer developed by Wiegand *et al.* for our further studies.

3.4 Conclusions

Aptamers are nucleic acid ligands that can recognize and bind to various target molecules with high specificity and affinity and have the ability to discriminate between closely related targets. They are amenable to easy chemical modification for fluorescent and enzymatic labeling and thus can be universal ligands that rival antibodies as tools in molecular recognition. To date, aptamers have been successively used instead of monoclonal antibodies in immunochemical sandwich assays, flow cytometry, and *in vivo* imaging (132) We attempted to screen high affinity binding ligands against HyHEL-5. HyHEL-5 as an immunoglobulin G prototype has various applications in basic research. However, the aptamers that we screened did not show binding in either of the binding tests.

There are several factors that affect the success of selection high binding ligands. One of the limitations in the SELEX process is the mispriming of the random library that may result in truncated or extended products. Sometimes there is a dropout of potentially strong aptamers that are not competitive in amplification and less potent aptamers dominate in the process. For our next step towards aptamer screening, we plan to improve our SELEX conditions by increasing the stringency in the selection conditions, include counter-selection steps in each cycle that may help in getting better binders.

Chapter 4: **APTAMER-PHAGE REPORTERS FOR ULTRA-SENSITIVE
LATERAL FLOW ASSAYS**

4.1 Introduction

Allergy has become a major health concern in both children and adults. The term allergy is defined as a malfunction of the immune system in which an individual's immune system reacts to normally harmless substances called allergens in the environment. The allergens can be pollen, dust, certain foods that cause allergic reaction of the respiratory system and certain chemicals that can affect skin. Allergic reactions are unique because they induce excessive activation of mast cells and basophils by Immunoglobulin E (IgE) resulting in the release of inflammatory substances (e.g., histamine, platelet activating factor, neutrophil chemotactic factor of anaphylaxis, and leukotrienes). These substances are responsible for generating an inflammatory response, which can range from uncomfortable to dangerous conditions. These manifestations of allergy can range from low-grade atopic symptoms (eczema, asthma, and rhinitis) and malabsorption symptoms to acute life threatening anaphylaxis. Therefore the management and assessment of allergic disease is a growing and challenging field.

IgE has been demonstrated to be a mediator in diseases of type I hypersensitivity. Its key role in allergic reactions was first discovered by Ishizaka *et al.* (148) and Johansson *et al.* (149) in 1967. Since then, IgE has been known to play an important role in the allergic response, which is widely reported as a marker of atopic diseases, such as asthma, dermatitis and pollenosis (94). IgE has a very short half-life (~1 day) and its concentration in the serum is the lowest (0.3 µg/mL) of the five immunoglobulin subtypes (150). Despite its low concentrations, IgE is

extremely biologically active in the circulation. This is due to the fact that IgE binds to high-affinity receptors on the surface of mast cells and basophils, so that these cells may be highly sensitive to allergens even when the concentration of IgE in the circulation is very low. The levels of IgE become elevated in patients with atopic diseases, with the highest levels generally being seen in atopic dermatitis, followed by atopic asthma, perennial allergic rhinitis, and seasonal allergic rhinitis. An elevated total IgE level (>1000 ng/ml) is one of the major diagnostic criteria for allergic broncho-pulmonary aspergillosis (ABPA) (151). The level of total IgE in ABPA may be used to monitor disease activity and response to therapy. Total IgE level in serum is widely reported as a marker of atopic diseases (152). Serum IgE levels above 100 IU/mL (One IU/mL or kU/L is equal to 2.4 ng/mL (153), so this is 240 ng/mL) is an indication that a person is atopic (154). Therefore a rapid detection of IgE levels in serum samples is valuable for patients afflicted with allergy-mediated disorders. The conventional methods for the detection of IgE are the Radio-Allergosorbent Test (RAST) (155), the Enzyme-Linked Immunosorbent Assay (ELISA) (156) and the dot-blot ELISA. However, these traditional assays employ an antibody as the bio-recognition element that can suffer from limitations due to the inherent disadvantages of antibodies, such as instability to harsh conditions, limited possibility for modifications and selection in a biological system. Hence it is desirable to explore new bio-recognition elements for the detection of IgE with enhanced performance. In this respect, aptamers have proven to be

efficient alternative affinity reagents to antibodies and have been established as molecular tools in the field of analytical chemistry.

Aptamers are single stranded DNA/RNA oligonucleotides selected from large composite oligonucleotide libraries by Systematic Evolution of Ligands by EXponential enrichment (SELEX) (75,76). Their defined three-dimensional shape allows them to interact with a target molecule with high affinity and specificity. Compared with antibodies, aptamers exhibit properties that make them superior, such as high affinity, high flexibility, chemical stability, low production cost and ease of synthesis or modifications. Since the development of an aptamer to IgE by Wiegand *et al.* (97) in 1996, several aptamer-based applications have been reported for IgE detection such as capillary electrophoresis (CE) (157), fluorescence anisotropy (158) and Surface Plasmon Resonance (SPR) (159). In another study, aptamers have been used to couple with nanoparticles to develop higher sensitive labels for human IgE determination (160). In contrast to these techniques, immunochromatographic lateral flow methods utilizing viral nanoparticles based molecular detection offer advantages in terms of sensitivity and specificity. We have shown that enzymes and anti-analyte antibodies can successfully be coupled to these viral nanoparticles making them useful reporters (Chapter 2 of this dissertation, and (22)).

In this chapter, we describe the development of a novel aptamer-phage based immunochromatographic LFA to detect target analyte; this also serves to demonstrate the generality of the phage-LFA approach. M13 phage is employed as a

scaffold for the attachment of aptamers and enzyme reporters. The major coat protein of M13 phage offers numerous binding sites for reporters such as horseradish peroxidase or any affinity reagents. The protein gpIII of SAM-AviTag M13 phage also offers binding sites for analytes via avidin-biotin linkage. SAM-AviTag phage are derivatives of phage M13 where the N-terminus of the few-copy phage coat protein III contains the enzymatically biotinylatable AviTag peptide (GLNDIFEAQKIEWHE). The lysine residue (K) in the AviTag is a substrate for biotinylation by *E. coli* biotin ligase (BirA). Using streptavidin or neutravidin, any biotinylated affinity agent can then easily be linked to these enzymatically biotinylated phage particles. Here in this work, we employed AviTag phage as a scaffold for the attachment of aptamers via avidin-biotin linkage on protein gpIII while HRP is covalently attached on the major coat protein gpVIII. These modified AviTag bacteriophage particles with aptamers as bio-recognition elements are used as the basis of ultra-sensitive LFAs. We demonstrated the direct detection of spotted IgE and PBP2a (penicillin binding protein) of *Staphylococcus aureus* and developed a sandwich IgE assay based on capture of phage modified with anti-IgE aptamers and HRP on anti-IgE polyclonal antibodies on Fusion 5 LFA matrix. The experimental data demonstrate that this novel approach of utilizing aptamer-phage as reporters offers high sensitivity and specificity for target analytes. The implementation of aptamer as a bio-recognition element instead of an antibody yields a simple, cost-effective and highly sensitive framework for the detection of proteins.

4.2 Materials and Methods

4.2.1 Materials

Polyclonal anti-IgE was purchased from Fitzgerald Industries International (Acton, MA). Human IgE was obtained from Abcam (Cambridge, MA, USA). Mouse anti-lysozyme monoclonal HyHEL-5 antibodies were produced from hybridoma cells and purified by Protein G Affinity Chromatography by Biovest/National Cell Culture Center (Minneapolis, MN). The DNA aptamer for IgE (97) (5'-Biotin TEG GGGGCACGTTTATCCGTCCCTCCTAGTGGCGTGCCCC-3') was purchased from Integrated DNA technologies (IDT) (Coralville, IA, USA). It was synthesized with a biotin modification at the 5' end and with TEG (triethyleneglycol) as a long spacer arm (Figure 4-1). Biotin-TEG increases the oligo-biotin distance and potentially avoids possible hindrance issues in attaching the aptamers to the phage or any other nanoparticle. The IgE aptamer was dissolved in phosphate buffered saline, pH 7 (PBS) containing 1 mM $MgCl_2$ to achieve a stock concentration of 100 μM . Before its attachment to the phage, the aptamer was denatured by heating at 95 °C for 10 min and then renatured by gradually cooling it to room temperature (25 °C) over a period of one hour. This heating and cooling process promotes aptamer refolding for enhanced binding efficiency, and disrupts any pre-existing higher order structures that may interfere with the further functionalization process, allowing the biotin at the 5' end to interact freely with its avidin binding partner (161). As a control, a proprietary MRSA aptamer specific to *Staphylococcus aureus* PBP2a

(penicillin binding protein) was purchased from Base Pair Technologies (Pearland, TX). Its target protein PBP2a was purchased from Ray Biotech (Norcross, GA).

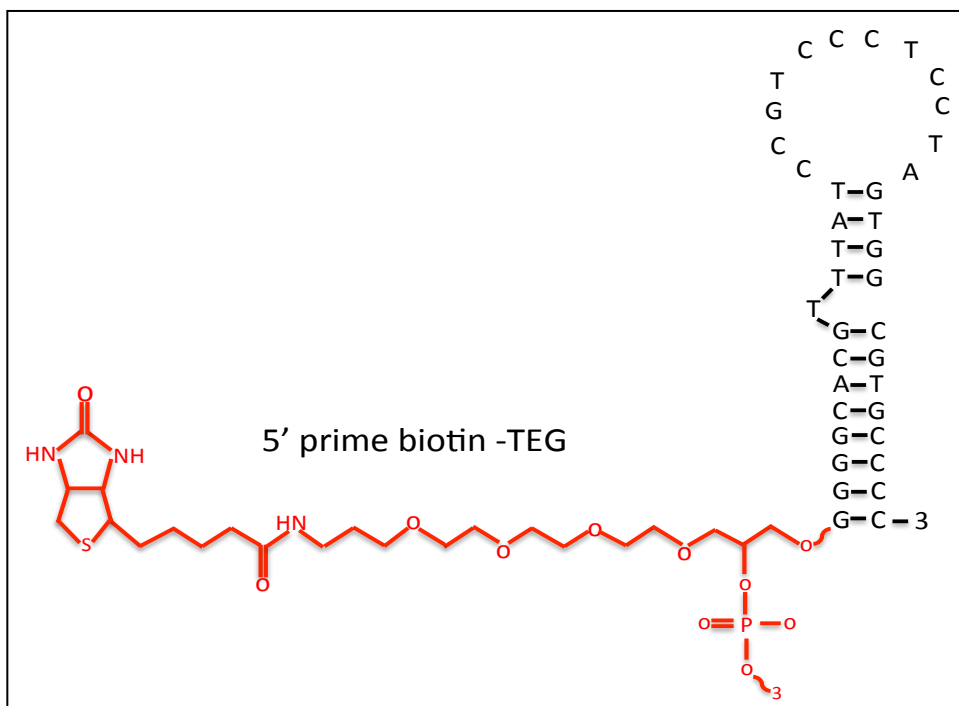


Figure 4-1: IgE aptamer with a 5' Biotin-TEG modification (11)

4.2.2 Validation of IgE aptamer in Enzyme Linked Oligonucleotide

Assay (ELONA)

Binding of the IgE aptamer to IgE protein was evaluated on a microtiter plate by ELONA (schematic shown in Figure 4-2). IgE was adsorbed on the standard ELISA plate (Medisorb, 96-well, NUNC) at concentration of 5 $\mu\text{g/mL}$ and incubated overnight at 4 $^{\circ}\text{C}$. The plate was washed three times with 0.1% PBS-Tween 20

(PBST) on a Tecan Hydroflex microplate washer, and blocked with 3% BSA. The plate was incubated for 2 hr at room temperature followed by three washes with PBST. Biotinylated IgE aptamer was first heat denatured at 95 °C for 10 min and then slowly re-natured at room temperature and thereafter various dilutions of biotinylated IgE aptamer (100 µL/well) were added. The incubation was continued for 2 hr at room temperature. The plates were washed thrice and then streptavidin-HRP (100 µL/well, 1:5000 diluted) was added. After 30 min of incubation the plate was washed again and 100 µL 1-Step™ Ultra TMB-ELISA Substrate (Thermo Scientific) was added to the wells. The reaction was stopped with 50 µL 1 M H₂SO₄ after 15 min and absorbance was measured at 450 nm in a Tecan Infinite M200Pro microplate reader.

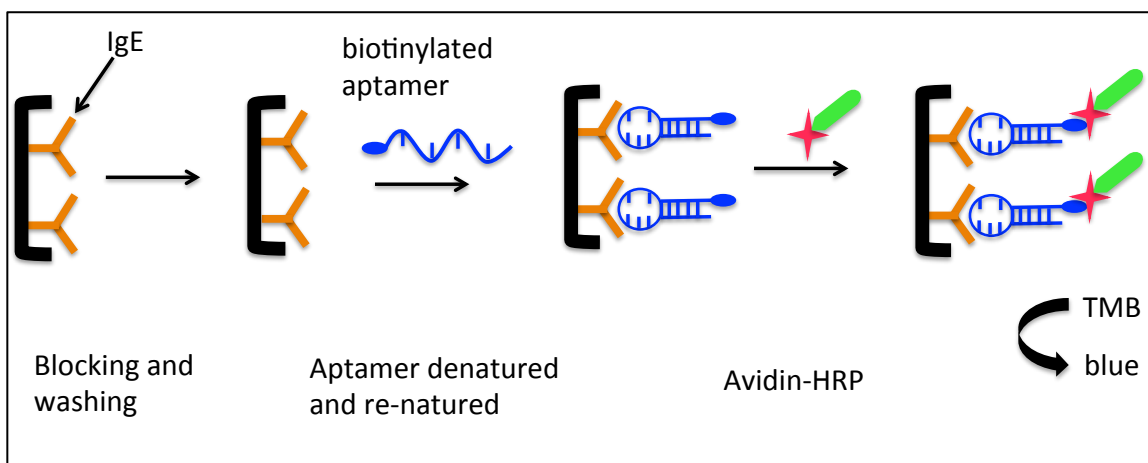


Figure 4-2: Scheme of Enzyme linked oligonucleotide assay (ELONA)

4.2.3 Propagation and titration of SAM-AviTag Phage

SAM-AviTag M13 phage were a gift from Dr. Brian Kay, UIC (Chicago, IL). For phage enumeration, *E. coli* TG1 was grown to mid-log phase in LB at 37 °C with shaking. The bacterial culture (5 mL) was then infected with 5 µL of phage stock ($\sim 10^{12}$ pfu/mL) and grown for 2 hr at 37 °C with shaking. This pre-culture was then transferred to 500 mL 2xTY medium and incubated overnight at 37 °C on a shaker. Bacteria were separated from phage in the supernatant by centrifugation (30 min, 3200x g) and filtration through 0.45 µm filters. Phage were then precipitated using PEG/NaCl as previously described (162). The concentrated phage were stored at 4 °C with 0.02% sodium azide. Phage titers were determined on X-Gal/IPTG plates as described (163).

4.2.4 Biotinylation of AviTag phage

The gpIII protein of SAM-AviTag phage was enzymatically biotinylated using *E. coli* biotin ligase (Avidity, Aurora, CO, or prepared in-house) as described in Section 2.2.4. The efficiency of biotinylation reaction was determined by ELISA on streptavidin-coated plates from StreptaWell High Bind, (Roche Applied Science, Indianapolis, IN) as described in Section 2.3.1.

4.2.5 Functionalization of M13 phage with IgE aptamer and HRP

After biotinylation of AviTag phage, HRP was chemically bound on the phage coat protein (gpVIII) and IgE aptamer was linked on the gpIII protein via avidin-biotin coupling (Figure 4-3). Briefly, 1 mL HRP (2 mg/mL, Sigma-Aldrich, St. Louis, MO) was covalently attached to the gpVIII coat protein of phage using Traut's reagent and sulfo-SMCC (succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate). 100 μ L of 1×10^{11} phage were suspended in 800 μ L PBS, pH 7.4, 3 mM EDTA with a 20-fold molar excess of Traut's reagent. This reaction was continued for 90 min at 25 °C on a rotator. Excess Traut's reagent was removed using 10 kDa filters (Millipore, Billerica, MA). Maleimide-HRP was separately prepared by mixing HRP and sulfo-SMCC in a final concentration of 22 μ M and 1.14 mM, respectively, and incubated for 30 min. Excess sulfo-SMCC was removed using a 10 kDa filter. Traut's reagent activated phage and maleimide-HRP were then incubated together for 90 min at 25 °C. Free HRP was removed using 100 kDa filters (Millipore). 1 μ L of 1 mg/mL of Neutravidin (Thermo Fisher Scientific) was offered to the HRP-labeled phage that bound through the biotinylated AviTag displayed on phage protein pIII. Free neutravidin was removed using 100 kDa filters (Millipore). 100 μ L of biotinylated IgE aptamer (100 nM) was first heat denatured at 95 °C for 10 min and then slowly re-natured at room temperature before it was added to the neutravidin-functionalized phage. The complex was incubated for 2 hr at 25 °C before the free IgE aptamer was removed using PEG precipitation. For the control experiments,

AviTag phage were also functionalized with the anti-PBP2a aptamer and HRP in the same way.

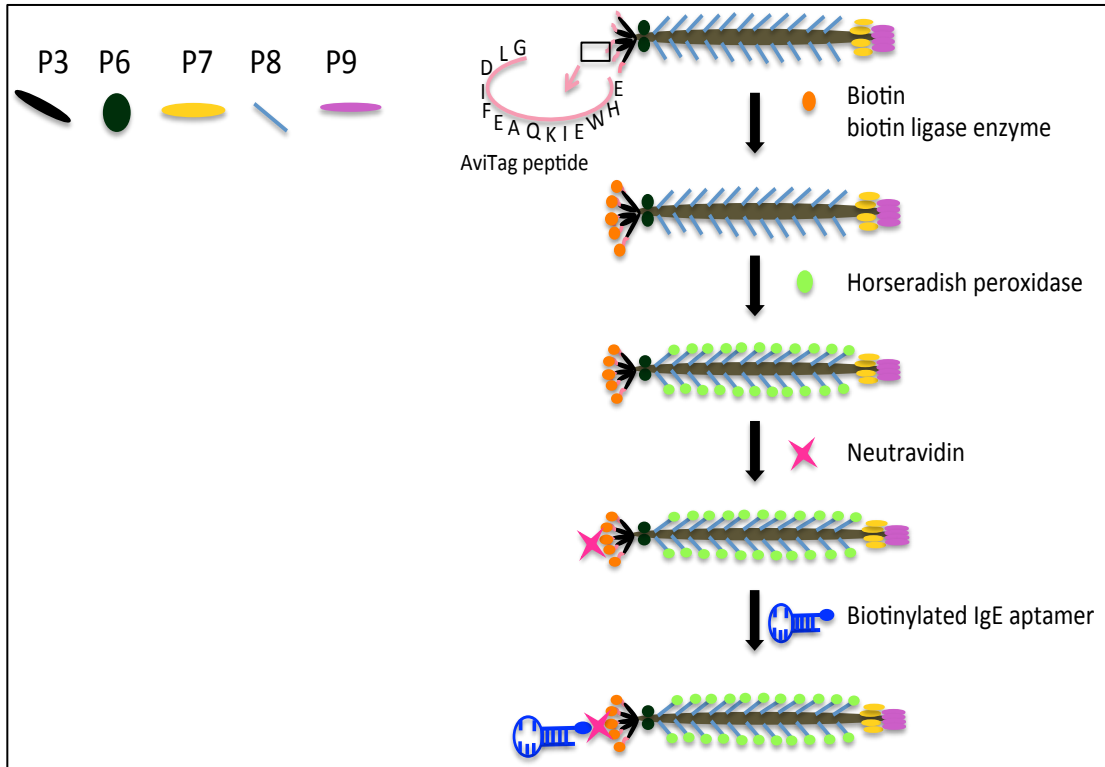


Figure 4-3: Schematic of functionalization of M13 phage with IgE aptamer and HRP

4.2.6 Determining the titer of the phage construct by PCR

After the functionalization of the phage, its concentration was determined by real-time PCR. A 10-fold serial dilutions (10^5 to 10^{10} phage/mL) of the phage were prepared from a stock whose titer had been determined using the classic double agar overlay plaque assay, and were used as standards in the real time PCR. The

number of functionalized apta-phage/mL was determined from these standards by real-time PCR using Brilliant III Ultra-Fast SYBR mix and primers specific for SAM-AviTag phage.

4.2.7 Lateral flow assay with apta-phage

4.2.7.1 Preparation of the LFA strips

5 μ L of polyclonal Anti-IgE (diluted to 100-fold) (Fitzgerald Industries International, Acton, MA) and 5 μ L of anti-M13 (GE Healthcare, Piscataway, NJ) (0.1 μ g/ μ L) antibodies in 50 mM sodium acetate buffer (pH 3.6) were used to make test lines and control lines on Fusion 5 membranes (GE Healthcare Biosciences, Piscataway, NJ) using a Lateral Flow Reagent Dispenser (Claremont BioSolutions). The strips were allowed to dry for 1 hr at 25 °C (schematic is shown in Figure 4-4).

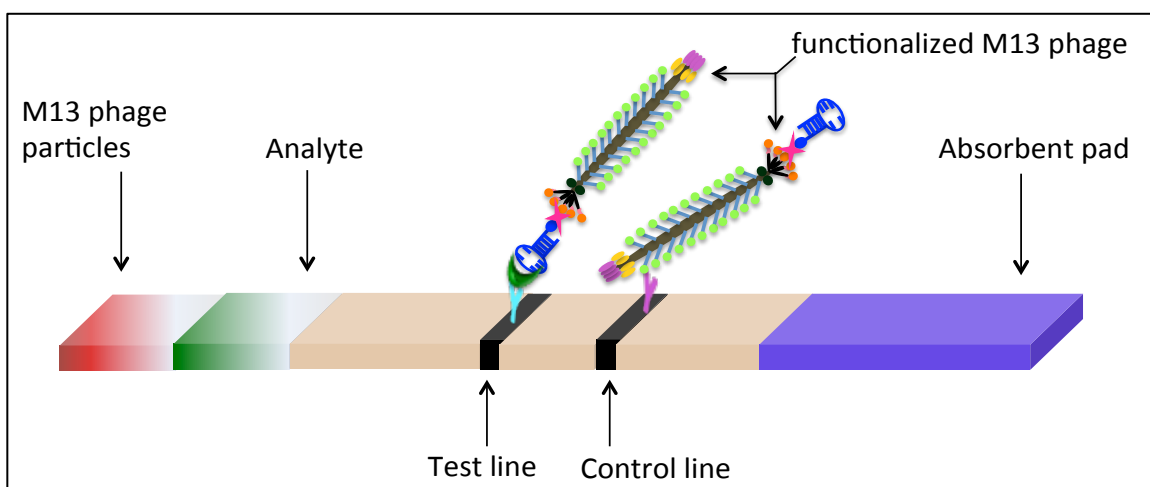


Figure 4-4: Scheme of apta-phage LFA

4.2.7.2 Direct detection of IgE protein and PBP2a protein by apta-phage construct

The initial LFA experiment was done to test the binding of the anti-IgE apta-phage and MRSA apta-phage directly to IgE and PB2a protein respectively on a Fusion 5 membrane. For this, 5 μ L IgE (0.2 mg/mL) and 5 μ L of anti-M13 (0.1 μ g/ μ L) in 50 mM sodium acetate buffer (pH 3.6) was spotted on the test and control lines, respectively, of a Fusion 5 membrane. For the MRSA apta-phage, 5 μ L PBP2a protein (0.2 mg/mL) was spotted at the test line with anti-M13 as the control line. The strips were dried for 1 hr at room temperature. Apta-phage constructs ranging from 10^7 to 10^9 were dispensed onto the distal end of the LFA strip. The strips were washed with 500 μ L LFA buffer (PBS, pH 7; 1% Tween 20; 1% Triton-X-100; 0.1% PEG-3350), before signals were obtained by spotting 25 μ L TMB Liquid Substrate System for Membranes (Sigma-Aldrich) on each line. The strips were scanned in a Perfection V600 flatbed color scanner. For quantitation of the data, the LFA strips were scanned evaluated using ImageJ's density analysis function (164).

4.2.7.3 Detection of IgE protein in the sandwich LFA

The IgE protein test analyte was diluted in PBS, pH 7 with 0.5% bovine serum albumin (BSA). 100 μ L aliquots each of the IgE samples were dispensed onto the sample application area of the prepared LFA strips. The strips were then washed with 200 μ L LFA buffer (PBS, pH 7; 1% Tween-20; 1% Triton-X-100; 0.1% PEG-3350) before 10 μ L of the phage construct ($\sim 10^9$ phage) were dispensed onto the

distal end of the LFA strip. The strips were washed again with 500 μ L LFA buffer. The signals were obtained as described in Section 4.2.7.2.

4.2.7.4 Different control experiments for the apta-phage LFA

To check for the specificity for the IgE detection by the apta-phage, various control experiments were performed. A control antibody, HyHEL-5, was spotted on the test line and then IgE protein followed by apta-phage were passed to see if IgE binds non-specifically to the unrelated protein. HyHEL-5 was also passed through the membrane in place of IgE to further check for non-specific binding. In another control, a competitive assay was done where first 13 ng IgE were passed through the membrane followed by the anti-IgE aptamer and the apta-phage construct, to see if IgE competes for binding.

As a control for the IgE apta-phage construct, a different apta-phage construct was made using a different aptamer (MRSA aptamer, specific for penicillin binding protein (PBP2a)). It was prepared in the same way as IgE apta-phage as a comparison for the method i.e. on the enzymatically biotinylated AviTag phage, HRP was chemically bound on the phage coat protein (gpVIII) and MRSA aptamer was conjugated on the gpIII protein via avidin-biotin coupling. This construct was passed on the membrane with anti-IgE and anti-M13 lines.

4.3 Results and Discussions

4.3.1 Binding of the IgE aptamer to IgE protein in ELONA

Prior to the apta-phage LFA, binding of the IgE aptamer to IgE protein was evaluated on a microtiter plate by ELONA. ELONAs are generally used to determine aptamer-binding affinities to their target. They can detect the presence of an analyte (by using aptamers as capture molecules) or detect the presence of aptamers (by using analytes as a capture molecules). IgE protein was used as the capture agent and adsorbed on the plate overnight at 4 °C. After several washing and blocking steps, different concentrations of the biotinylated IgE aptamer were added to the wells. Thereafter incubation and further washing steps, streptavidin-HRP-substrate was added for detection followed by 1-Step™ Ultra TMB-ELISA substrate. The reaction was stopped with 50 µL of 2 M H₂SO₄, and the absorbance was measured in the plate reader at the wavelength of 450 nm. The results in the graph in Figure 4-5 show that there is an increase in the absorbance at 450 nm with an increase in the IgE aptamer concentrations, thus confirming that the IgE aptamer binds to the immobilized IgE protein. The control well with no aptamer shows a relatively low absorbance as compared to wells with aptamers, thus indicating the proper functioning of the assay.

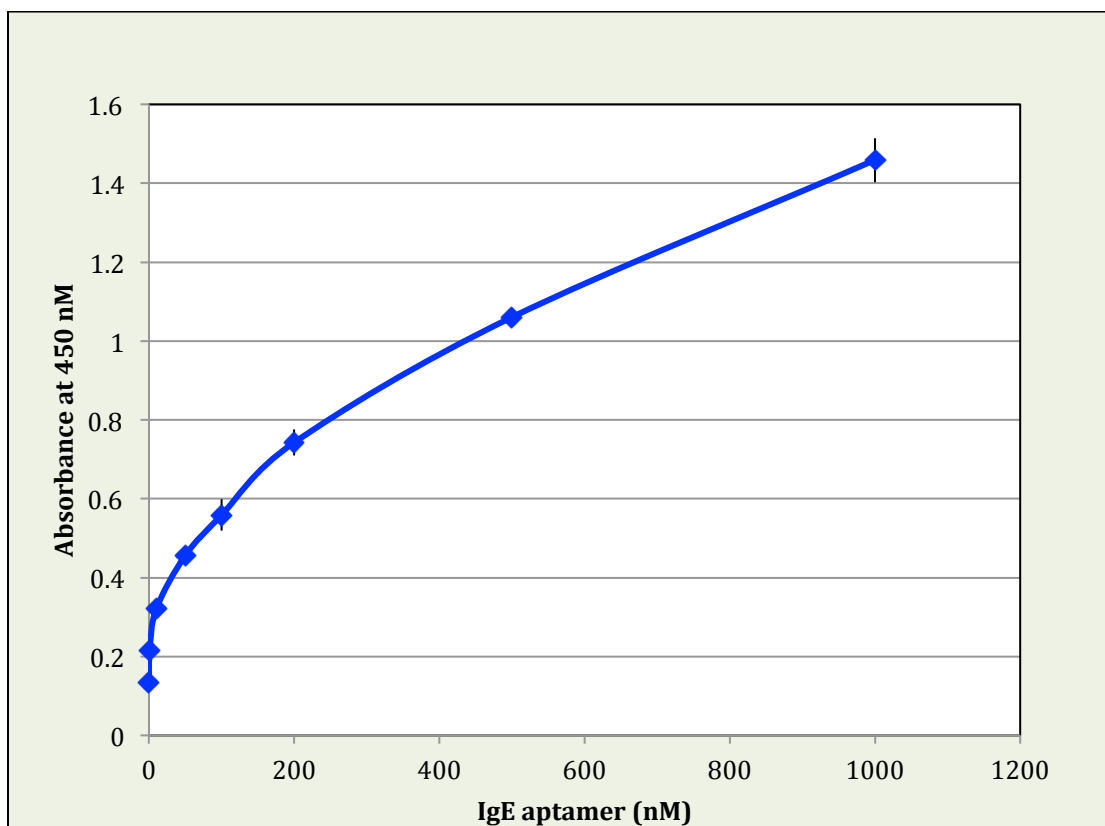


Figure 4-5: Absorbance obtained at 450 nM in the ELONA showing binding of IgE aptamer to IgE protein

Each point represents the mean of two determinations. The range of the assay was from 1 nM to 1000 nM.

4.3.2 Evaluation of the apta-phage for binding to IgE and PBP2a by direct detection in LFA

Different concentrations of apta-phage (10^7 , 10^8 and 10^9 phage/mL) were dispensed on the distal end of the LFA membrane with IgE, PBP2a test lines and anti-M13 control lines. The strips were washed with 500 μ L LFA buffer and signals were

obtained by spotting 25 μL TMB Liquid Substrate System for Membranes on each line. As shown in the Figure 4-6, a clear signal is obtained at the anti-M13 control line for all samples, thus confirming that the apta-phage moved successfully through the membrane. At the test line, the signal obtained for 10^9 apta-phage is clear and distinguishable from the other apta-phage concentrations. The signals for 10^8 and 10^7 are weak. This indicates that apta-phage LFA requires at least 10^9 apta-phage particles to obtain clear and distinguishable signal. The strip with HRP-M13 phage (i.e., without phage without the anti-IgE aptamer) does not give any signal on the test line thus indicating that M13 phage without IgE aptamer is not retained non-specifically by IgE, but gives a signal on the control line.

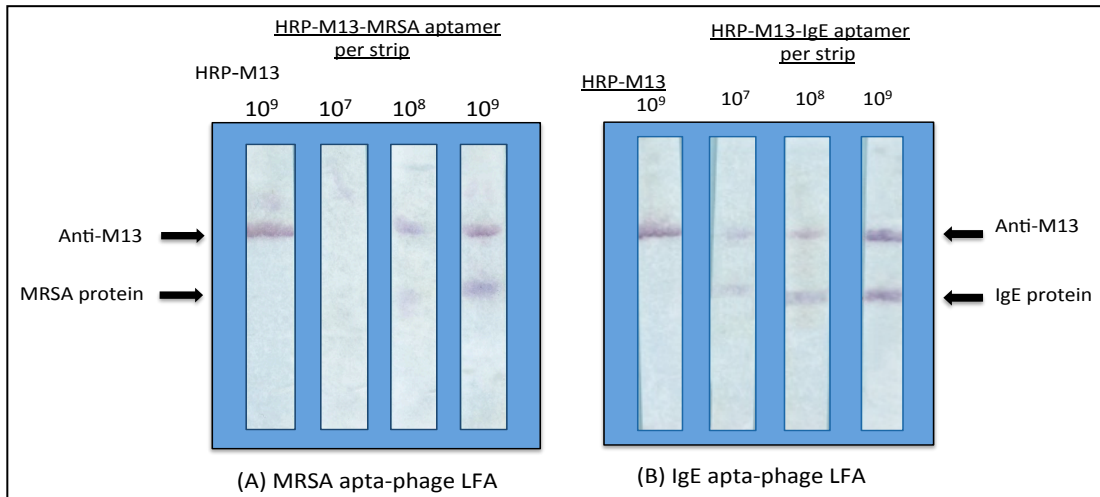


Figure 4-6: Direct detection of target analytes with apta-phage.

(A) IgE and anti-M13 antibodies were spotted on the test and control lines, respectively. (B) PBP2a and anti-M13 antibodies were spotted on the test and control lines, respectively. Different concentrations of apta-phage constructs in 100 μL LFA buffer were then passed through the membrane. AviTag phage carrying just the HRP reporter (HRP-M13) enzyme served as a control.

4.3.3 LFA with apta-phage for IgE detection

A sandwich IgE assay based on capture of phage modified with anti-IgE aptamers and HRP on anti-IgE polyclonal antibodies on Fusion 5 LFA matrix was developed. Serial dilutions of the IgE test analyte were diluted in PBS, pH 7 with 0.5% bovine serum albumin (BSA). 100 μ L of each IgE sample were dispensed onto the sample application area of the prepared LFA strips. To capture phage-bound IgE, anti-IgE antibodies were spotted at the detection line. As seen in Figure 4-7 shows, a signal is obtained at the anti-M13 control line for all samples suggesting that the test has performed correctly and the apta-phage particle reporters had moved through the membrane. A signal clearly distinguishable from the control containing no IgE is obtained on the strip with 0.13 ng/mL of IgE on the test line (Figure 4-7A). In a sample volume of 100 μ L, this corresponds to a concentration of 0.684 pM. For quantification purposes, ImageJ density analysis function was performed (Figure 4-7B). Table 4-1 shows the relative intensities of the test and control lines of the strips in Figure 4-7. The relative intensities of the test lines correspond to the amount of IgE detected by the apta-phage, and it agrees well with the signals obtained on the strip by simple visual inspection. The relative intensities of the test lines were divided by the control lines (T/C) to adjust for any background variation. The apta-phage yielded an excellent correlation between the T/C values and the IgE concentrations, which is desirable for quantification. The limit of detection (LOD) was evaluated to be 0.13 ng/mL based on ImageJ density analysis function. This is

~100-fold more sensitive than the commercial ELISA kit for quantification of IgE (The detection limit of the commercial ELISA kit from Abcam is 12 ng/mL and for that from ebioscience Inc. (San Diego) is 7.8 ng/mL (99) and 230-fold more sensitive than the previously reported data (99).

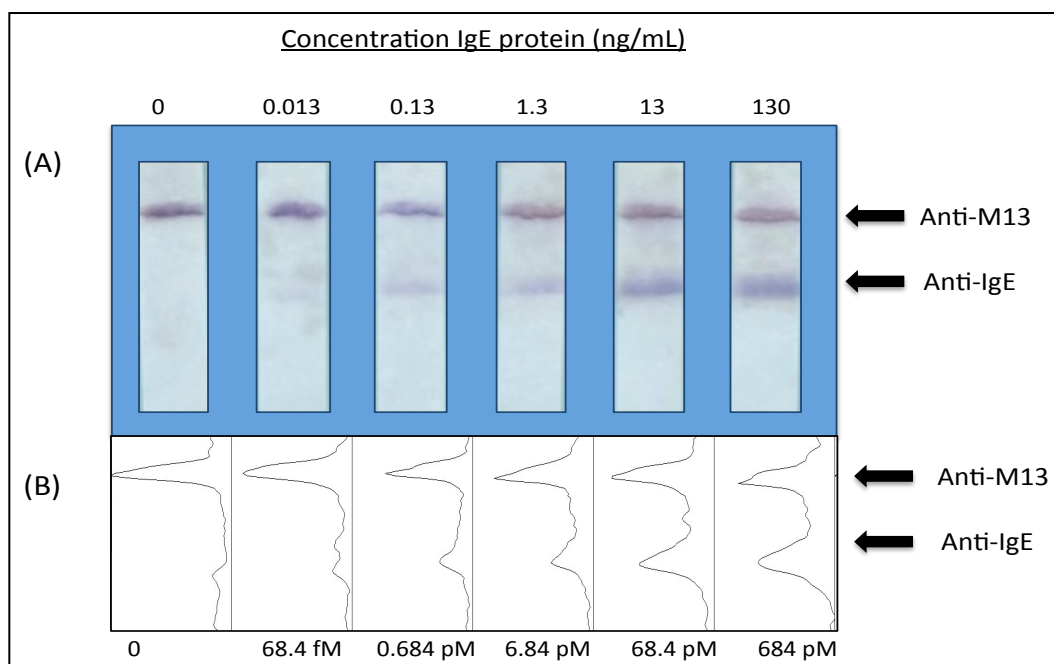


Figure 4-7: Aptaphage LFA to detect IgE

(A) Various concentrations of IgE in 100 μ L LFA buffer were passed through an LFA membrane with anti-IgE antibodies as the test line, and anti-M13 antibodies as the control line. Phage functionalized with HRP and biotinylated IgE aptamers ("aptaphage") were added to bind to both the test (in the presence of analyte) and control lines. (B) Plots of test and control lines of the LFA strips in Figure 4-7A as determined by ImageJ density analysis (molar concentrations of IgE are also shown below each plot).

Table 4-1: Relative intensities of the test lines and control lines shown in Figure 4-7 as determined by ImageJ

IgE (ng/mL)	0	0.013	0.13	1.3	13	130
Control line	5726	6526	3878	4326	4741	3928
Test line	0	0	743	1901	4092	4826
(Test/Control)	0	0	0.19	0.44	0.86	1.23

4.3.4 Control experiments for the apta-phage LFA

4.3.4.1 Specificity of the apta-phage

To verify the specificity of the anti-IgE apta-phage, a different antibody, HyHEL-5, was spotted on the test line with anti-M13 antibodies on the control line. IgE was passed through the membrane followed by washing steps and apta-phage reporters. As seen in Figure 4-8, there is no signal on the HyHEL-5 test line when 130 ng of IgE was passed through, thus indicating that IgE did not bind to HyHEL-5 on the test-line. In addition, no non-specific binding of apta-phage to HyHEL-5 was seen (the strip with no IgE where only apta-phage was passed). The control lines yielded a clear signal on both strips, confirming proper functioning of the assay. Results of the ImageJ analysis density function comply with the visual inspection of the strips (Figure 4-8 and Table 4-2).

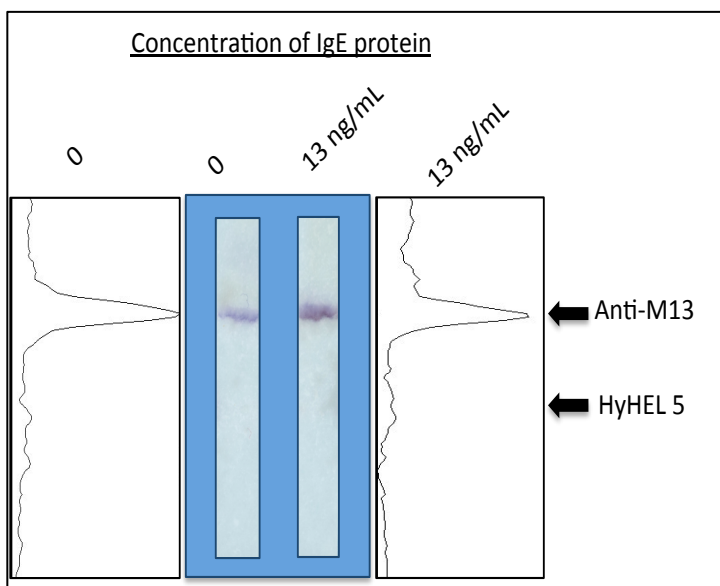


Figure 4-8: Control for non-specific binding of IgE and apta-phage to an unrelated protein

HyHEL-5 was spotted on the test line and anti-M13 antibodies on the control line. IgE (100 μ L in LFA buffer) was passed through the membrane and apta-phage were added as reporters. Plots of ImageJ density analysis of the LFA strips are shown on the sides of the Figure 4-8.

Table 4-2: Relative intensities of the test lines and control lines shown in Figure 4-8, as determined by ImageJ

IgE (ng/mL)	0	13
Control line	2250	5801
Test line	0	0
Test/Control	0	0

4.3.4.2 Specificity of the apta-phage LFA

The apta-phage LFA was evaluated for non-specific binding to other proteins. The specificity was evaluated by comparing HyHEL-5 and IgE binding on LFA membrane with anti-IgE and anti-M13 (as test and control lines) followed by apta-phage capture of target. HyHEL-5 was passed through a membrane with anti-IgE and anti-M13 antibodies on one strip. Various concentrations of IgE were passed through other strips for comparison (as shown in Figure 4-9). The figure clearly depicts that HyHEL-5 did not bind to anti-IgE since no signal is observed on the test line. This is also confirmed through the quantitative data obtained from the ImageJ density analysis in Figure 4-9A and Table 4-3. The strips where IgE was added reveal that IgE binds specifically to the anti-IgE test line, followed by the retention of functionalized apta-phage, hence demonstrating the efficient function of the actual apta-phage LFA for IgE detection. A clear signal is observed on the control line indicating that the apta-phage binds on the anti-M13 antibody and hence assay the assay works well.

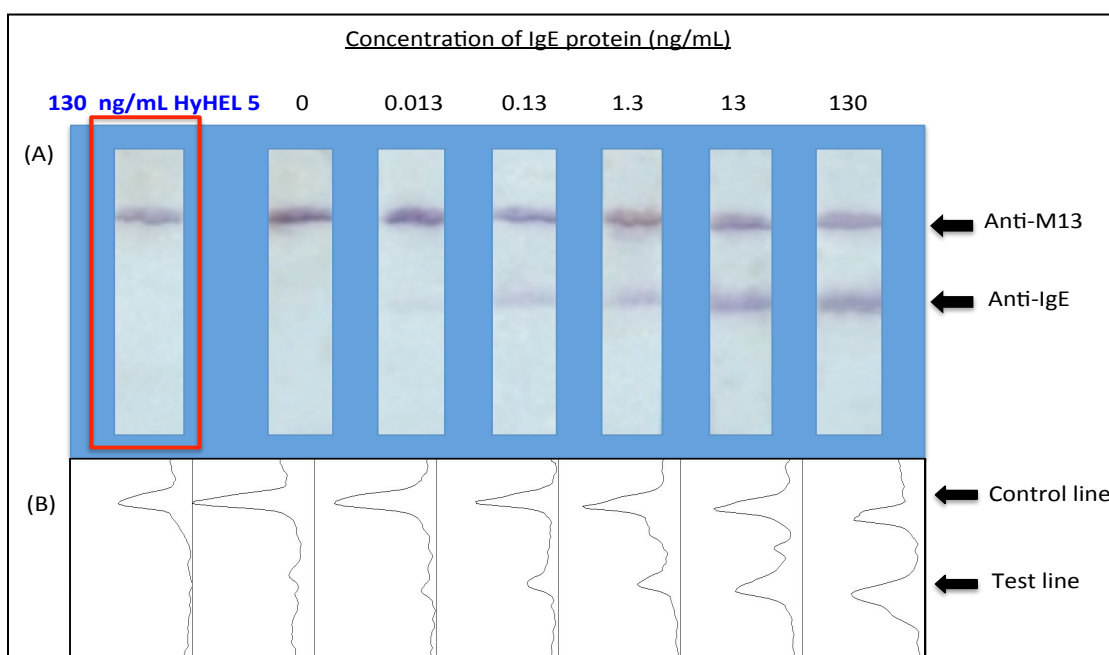


Figure 4-9: Evaluation of non-specific binding of apta-phage to unrelated proteins

(A) 100 μ L in LFA buffer HyHEL-5 was passed through the membrane with anti-IgE and anti-M13 lines. Various concentrations of IgE proteins (100 μ L in LFA buffer) also passed on the membrane. IgE binds on the anti-IgE test line (as shown in the other strips). Signals on the control lines indicate the proper functioning of the assay. (B) ImageJ density analysis of the strips of Figure 4-9A.

Table 4-3: Relative intensities of the test lines and control lines shown in Figure 4-9 and as determined by ImageJ

Protein (ng/mL)	HyHEL-5	0	0.013	0.13	1.3	13	130
Control line	3184	4171	5336	3445	4730	3632	2765
Test line	0	0	0	1061	1338	3089	5614
Test/Control	0	0	0	0.31	0.28	0.85	2.03

4.3.4.3 Competition assay (Saturating IgE protein with soluble IgE aptamer)

In this control, anti-IgE antibodies were spotted on the test line and anti-M13 antibodies on the control line. IgE (130 ng/mL) was passed through the membrane, followed by an excess amount of soluble IgE aptamer to saturate the binding sites on the IgE protein. Finally, anti-IgE apta-phage reporters were added to see if any are retained on the test line. The results are depicted in the Figure 4-10. IgE binds on the anti-IgE test line (as shown in the other strips that did not contain any free anti IgE aptamer). When soluble aptamer is passed, it should bind to the IgE protein leaving no space for the apta-phage construct on the bound IgE on test-line. Therefore when the apta-phage construct is added, we do not see any signal, as expected. The ImageJ density analysis and relative intensities of the strips are also shown in the Figure 4-10B and Table 4-4.

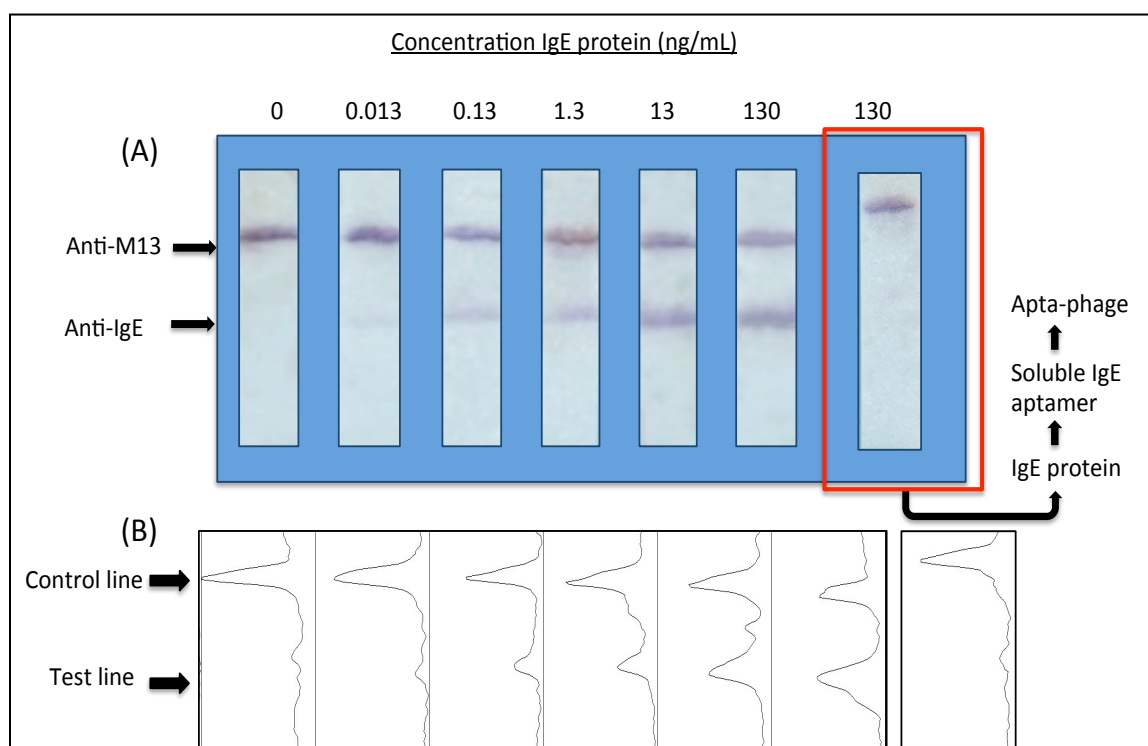


Figure 4-10: Saturating IgE binding sites with soluble IgE aptamer

(A) 130 ng/mL IgE in 100 μ L of LFA buffer was passed (strip with Red boundary), followed by soluble IgE aptamer and then final apta-phage construct on the anti-IgE and anti-M13 lines. (B) ImageJ density analysis of the strips in Figure 4-10(A).

Table 4-5: Relative intensities of the test lines and control lines shown in Figure 4-10 as determined by ImageJ

IgE (ng/mL)	0	0.013	0.13	1.3	13	130	130 ng/mL aptamer
Control line	5024	5721	3562	4117	4159	3718	3574
Test line	0	0	998	1725	3650	4252	0
Test/Control	0	0	0.28	0.42	0.87	1.14	0

Immunochromatographic assays are a useful analytical method for point-of-care diagnostics but still demand improved analytical sensitivity. This has been addressed in this work by developing improved viral nanoparticle reporters. To achieve this we employed M13 phage as a scaffold for attaching multiple reporter enzymes on the major coat protein (gpVIII) and avidin-mediated coupling of biotinylated IgE aptamer to enzymatically biotinylated AviTag peptides displayed on the phage tail proteins. The single coat protein gpVIII bears five lysines for reactive primary amines, but among them, only one lysine is easily accessible for modification. Even with this, the 2700 gpVIII coat proteins of phage M13 offer hundreds of binding sites for horseradish peroxidase thus obtaining a stronger signal per individual affinity agent.

4.3.4.4 MRSA-apta-phage as control for IgE-apta-phage reporters

In this control experiment, another apta-phage (MRSA-phage) was used instead of the anti-IgE- phage construct on the membrane with anti-IgE and anti-M13 lines. MRSA aptamer is a proprietary aptamer specific to *Staphylococcus aureus* PBP2a (penicillin binding protein). It is a DNA aptamer that was selected from a randomized 32-mer library against MRSA protein. Since IgE aptamer is a DNA aptamer, we selected a DNA aptamer (MRSA) as an appropriate control for the apta-phage LFA. The MRSA apta-phage construct was made in a similar way as the IgE apta-phage construct.

The strip with the red boundary is shown in Figure 4-11. Here first 13 ng of IgE in 100 μ L of LFA buffer is passed followed by MRSA-phage. As expected, the anti-MRSA aptamer on the phage did not bind to the IgE protein on the test line and hence there is no signal on the test line. As a control for the MRSA-phage construct, strips with MRSA protein and anti-M13 on the test and control lines, respectively, were made [shown in the black boundary]. A clear signal is obtained on the strip where MRSA-phage were passed indicating that the MRSA-phage bind to the MRSA protein. The strip with HRP-phage (only HRP-phage construct without MRSA aptamer) label did not show any signal, indicating that phage do not just bind non-specifically to the MRSA protein. The test and control lines were quantified by ImageJ density's analysis function. The plots and relative intensities of test and control lines are depicted in Figure 4-11B and Table 4-5, respectively. These results suggest that the apta-phage have selectivity towards IgE and exhibits no non-specific binding.

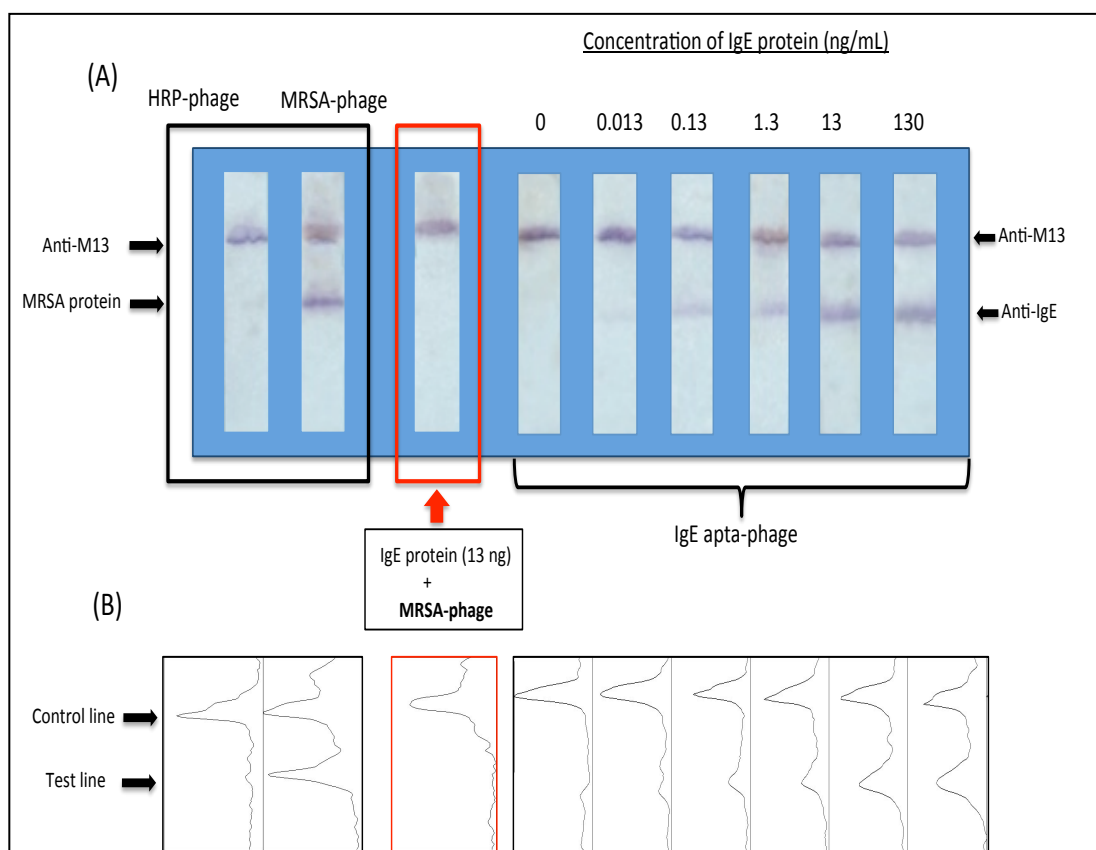


Figure 4-11: Control for IgE aptamer on phage

As control for the apta-phage construct (IgE-M13-HRP), another apta-phage construct was made using a different aptamer (MRSA aptamer, specific for penicillin binding protein (PBP2a)). This construct was passed on the membrane with anti-IgE and anti-M13 lines. Strips with MRSA protein and anti-M13 on test-line and control line were also made to evaluate the binding of MRSA-phage to MRSA protein. (B) Plots of test and control lines from ImageJ analysis of strips from Figure 4-11A.

Table 4-5: Relative intensities of the test lines and control lines shown in Figure 4-11A and as determined by ImageJ

IgE protein (ng/mL)	MRSA-phage+IgE protein	0	1.3 pg	13 pg	130 pg	1.3 ng	13 ng
Control line	3905	5048	6191	3675	4474	4548	3004
Test line	0	0	0	9250	1634	3438	6523
Test/Control	0	0	0	0.25	0.36	0.75	2.17

MRSA protein	HRP phage+MRSA protein	MRSA-phage+MRSA protein
Control line	4686	3247
Test line	0	4358
Test/Control	0	1.34

4.4 Conclusions

We have demonstrated a novel, sensitive and highly specific method for the determination of proteins using viral nanoparticles as scaffolds for conjugating aptamers as bio-recognition element. The conjugation of the IgE aptamer to the pIII protein of the phage via avidin-biotin coupling and multiple enzymatic reporters on the major phage coat protein constitutes a novel framework for detecting IgE and other proteins such as MRSA in lateral flow assays. Compared to the previously reported LFA work, our phage based assay method showed greater sensitivity in detection of proteins such as IgE. The novel apta-phage method showed a reduction in LOD by 230 times as compared to previously reported methods (99) and about

100 times compared to a (rather insensitive) commercially available ELISA kit. The use of apta-phage as reporters in lateral flow assays provides a convenient, cost-effective and specific framework for the detection of analytes. It employs aptamers as bio-recognition element instead of antibodies. Compared with antibodies, aptamers have high affinity, increased chemical stability, low production cost and can be easily synthesized or modified that make them better bio-recognition element over antibodies. The 2,700 copies of the coat protein of M13 phage provide multiple sites for enzyme conjugation such as HRP, thus providing a stronger signal per individual affinity agent. This novel and cost-effective approach of the apta-phage based LFA method provides great promise in protein analysis, particularly for very low-level detection of target analyte.

Chapter 5: **CONCLUSIONS AND FUTURE DIRECTIONS**

5.1 Functionalized viral nanoparticles as ultrasensitive reporters in lateral-flow assays

Phage nanoparticles offer versatile scaffolds that can accommodate a variety of recognition and reporter elements with high colloidal stability thus making them universal and ultrasensitive bio-detection reporters. We demonstrated that M13 phage decorated with peroxidase enzymes and anti-analyte antibodies can serve as reporters in lateral-flow assays of greatly enhanced sensitivity. The 2,700 gpVIII coat proteins of phage M13 offer a multitude of binding sites for reporters such as horseradish peroxidase and affinity agents such as antibodies or aptamers specific for the target of interest. In addition, the gpIII protein of a modified AviTag M13 phage also provides binding sites for analytes via avidin-biotin linkage. We compared both approaches for the development of improved reporter particles: the covalent linkage of the antibodies to the phage coat proteins and the avidin-mediated coupling of biotinylated antibodies to enzymatically biotinylated AviTag peptides displayed on the phage tail proteins. These modified M13 particles were able to detect MS2 virus in lateral flow assays with great sensitivity (with the limit of detection 10^4 pfu/mL) and specificity. Both the approaches gave almost one thousand-fold increased sensitivity compared to gold nanoparticle LFA with the same antibodies. We did not observe any significant difference in sensitivity between M13 phage containing anti-MS2 antibodies attached to the gpIII tail protein, and those with the antibodies attached to the phage coat. Assuming that the

number of gpIII molecules is between three and five per phage, and the number of pVIII coat proteins is 2700, we would have expected a significantly larger number of antibodies per phage in the latter approach.

We believe that the success of M13 phage reporters with few antibodies agrees well with monovalent phage display format. In this format, the phage are retained at significant efficiency by affinity of a single displayed antibody and hence argues that M13 LFA reporters should display small numbers of antibodies and a maximal number of enzyme reporters (165,166). We are actively pursuing the optimization of the assay, including its integration into a paper microfluidics system that will reduce the number of hands-on steps. In the future, we also plan to investigate the exact effect of varying the number of functionalized viral nanoparticles used in the assay and the number of enzymes and antibodies per viral particle. We are further optimistic that the use of reporter enzymes other than HRP (with or without the combination with chemiluminescent substrates) will yield higher sensitivity. Lastly, we are investigating functionalized viral nanoparticles with different morphologies (e.g., TMV, T7 phages) as alternative reporters. We plan to examine the use of alternative enzyme reporters and substrates for our LFA scheme, especially chemiluminescent substrates for future, and extend the use of this approach to detection of pathogenic viruses in blood serum. Immunochromatographic assays are a widely useful method for point-of-care diagnostics but still suffers the extreme sensitivity as compared to ELISAs and PCRs. Phage LFA shows a superior sensitivity over conventional lateral flow assays and we

anticipate that it can have an immediate impact in the areas of point-of-care detection.

5.2 Towards the Isolation of DNA aptamers specific to murine IgG HyHEL 5

Antibodies, the most popular class of biomolecules have a wide range of applications in basic research, diagnostics and therapeutics. They provide molecular recognition needs for a wide range of applications. The ability to recognize their antigens with great specificity has made them powerful tools in basic research. They have the ability to selectively bind a broad range of analytes with high affinity and thus have made substantial contributions toward the advancement of several diagnostic assays and thus have become indispensable in the diagnostic areas. However, there are certain limitations associated with antibodies, for example, they are sensitive to temperature and prone to irreversible denaturation (132). The production of antibodies requires animal sources that can suffer from batch-to-batch variation. In addition, antibodies have high molecular weight and it is difficult to achieve site-specific labeling. Furthermore antibody-based assays usually require immobilization and extensive washing.

Alternatively, it is possible to consider an entirely different class of ligands, i.e., aptamers, to circumvent the shortcomings of antibodies. Their small size, high stability, (especially DNA aptamers), high binding affinity and specificity, and ease of

modification make them attractive tools in analytical chemistry. DNA aptamers are more stable than proteins and can be repeatedly used without losing their binding capability. Aptamers have little immunogenicity or toxicity. Their small size, offers a higher density for immobilization and binding to epitopes that are not easily accessed by antibodies. Aptamers could serve as a valuable tool in the diagnostic area, as bio-recognition elements in various immunoassays such as in immuno-PCRs and LFAs.

Here, we attempted to screen DNA aptamers specific to HyHEL 5. HyHEL 5 is an immunoglobulin G (IgG) that is required for various research purposes. Our goal was to make DNA aptamers against that can be used as model for various applications such as in diagnostics, as recognition elements or as labels in immuno-PCRs. Ten rounds of SELEX were performed to select high affinity ligands against HyHEL 5. A few selected aptamers were tested for binding to HyHEL 5 by ELONA and SPR analysis. As a positive control a known RNA IgG aptamer was also tested. However, the resulting aptamers were unable to show successful binding to HyHEL 5. Since the selected DNA aptamers did not show successful binding to the target, a well-studied DNA aptamer against IgE was employed in the apta-phage studies.

The success of screening high binding ligands in SELEX process depends on various factors such as temperature, salt concentrations, pH, and washing. Sometimes, functionally less potent molecules, may get overrepresented due to the fact that they have the ability to replicate slightly better during PCR than their competitors. Moreover, there is no standardized SELEX protocol for all targets. The

selection conditions depend on the current circumstances such as target, desired features of the aptamers, and applications. In the future, we plan to improve our SELEX conditions by increasing the stringency, include counter-selection steps in each cycle that may help in getting successful binders.

We further plan to select DNA aptamers against the Fc region of IgG apart from the whole IgG. At present, protein A affinity chromatography is used for the purification of therapeutic antibodies, because it can selectively bind the Fc region of variety of mammalian IgG molecules (167). Since Protein A is a natural product of *Staphylococcus aureus*, it can cause immunogenic or other physiological problems in humans, if any contaminating ligand is leached from the matrix. Alternatively, aptamers selected against Fc portion of IgG will be generally useful reagents for the affinity purification.

5.3 Aptamer-phage reporters for ultra-sensitive lateral flow assays

In this dissertation, we presented the first modification of bacteriophage particles with aptamers as bio-recognition elements and demonstrated the use of these particles as the basis of ultra-sensitive lateral flow assays. M13 AviTag phage is utilized as a scaffold for the attachment of aptamers via avidin-biotin linkage while HRP is covalently attached on the major coat protein gpVIII. We demonstrated the direct detection of spotted IgE and PBP2a of *Staphylococcus aureus* and developed a

sandwich IgE assay based on capture of phage modified with anti-IgE aptamers and HRP on anti-IgE polyclonal antibodies on Fusion 5 LFA matrix. The limit of detection of this LFA was 0.13 ng/mL which is ~100-fold sensitive than the commercial ELISA and ~200 fold sensitive than the previous reported IgE detection assays (99). These AviTag phage-based LFAs are extendable to any biotinylatable affinity reagent. The use of apta-phage as reporters in LFAs provides a very simple, cost-effective and highly specific framework for the detection of proteins since it employs aptamers as bio-recognition element instead of antibodies.

Immunoglobulin E (IgE), found only in mammals, plays a key role in allergic responses and is capable of triggering the most powerful immune reactions. It is widely reported as a marker of atopic diseases. Therefore, detection of IgE is of great interest in dealing with patients afflicted with allergy-mediated disorders. In future, we propose to extend the use of this apta-phage approach to the detection in clinical samples. We also plan to explore the use of alternative reporters and substrates for our LFA scheme, especially the use of fluorescent dyes such as Alexa 555 and FITC. As a next step we are actively in the pursuit of developing a complete sandwich lateral flow assay for the PBP2a protein by MRSA aptamer. We will also explore the use of standard nitrocellulose membrane in our lateral flow methods.

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