

12-2011

# In Vitro Selection of Aptamers Against Avian Influenza Virus H5N1

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IN VITRO SELECTION OF APTAMERS  
AGAINST AVIAN INFLUENZA VIRUS H5N1

IN VITRO SELECTION OF APTAMERS  
AGAINST AVIAN INFLUENZA VIRUS H5N1

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Cell and Molecular Biology

By

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December 2011

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## ABSTRACT

Over \$10 billion losses in the poultry industry were caused by avian influenza (AI) so far. Rapid and specific detection of avian influenza virus is urgently needed with the concerns over the outbreaks of highly pathogenic H5N1 influenza virus and cases of animal and human infection. Aptamers are oligonucleic acid or peptide molecules that bind a specific target molecule with good affinity. They show better thermal stability than antibodies. The goal of this research was to select DNA-aptamers as the specific recognition element of AI H5N1 virus to be used in detection assays specific for field application. In this study, Systematic Evolution of Ligands by EXponential enrichment (SELEX) was used to select DNA aptamers targeted to hemagglutinin (HA) and neuraminidase (NA) proteins of AI H5N1 virus. In the first four cycles of selection, aptamers were selected by incubating HA proteins with a DNA library starting from  $10^{14}$  molecules randomized at central 74 nt and subsequent nitrocellulose filtration. Then aptamers were eluted from filters and amplified by PCR. Single stranded DNA aptamers were derived from these double stranded DNAs by  $\lambda$  digest and were used as input for the next selecting cycle. In the following 9 cycles of selection, H5N1 virus was incubated as a substitute of NA proteins with aptamers pool in SELEX process. After 13 cycles of isolation, 115 bp DNA-aptamers were screened out and three aptamer sequences were obtained after cloning. Results of Dot ELISA and Dot Blot showed that these DNA-aptamers have stronger binding specificity and affinity to AI H5N1 subtype compared with their binding to H5N2, H5N3, H5N9, H2N2, H7N2 and H9N2. SPR test detected binding affinity of aptamer O16 to HA protein  $K_D$  is  $4.65 \times 10^{-9}$  M and a linear equation between y (SPR signal) in RU and x (virus titer) in HAU was described as:  $y=208.39x + 2.2347$  ( $R^2=0.99$ ). But SPR results showed aptamers had weak cross-reaction with H5N2. These selected aptamers could be applied to detection of AI H5N1 virus in the future.

This thesis is approved for

Recommendation to the

Graduate Council

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## ACKNOWLEDGEMENTS

I would like to express the deepest appreciation first to my advisor Dr. Yanbin Li for his continuous support in my master program. As a research professor, he taught me to work hard and think critically on my research. I also learned the spirit of working independently and cooperating together with the whole group from him. As a graduate advisor, he guided us not only in academic area but also in personal development. His own motivation in his academic life always encouraged me to pursue higher and higher goals. I would like to especially thank Dr. Li's help during my difficult times; I would not accomplish my research work and thesis without his persistence in helping me out from difficulties.

Unique thanks goes to another two professors in my advisory committee, Dr. Gisela F. Erf and Dr. Young Min Kwon. Dr. Erf taught me the lecture 'advanced immunology' and pointed out some problems during my research period and showed me guidelines to solve them. Dr. Kwon guided me in molecular biology area where I learned large amounts of techniques from this specialist. Meanwhile, Dr. Kwon also provided some instruments and reagents I needed in my research. Thanks to Dr. Kwon's whole lab group for their kind help.

I will also give my sincere thanks to Dr. Ronghui Wang, Dr. Chuanmin Ruan, and Dr. Tieshan Jiang. They directly guided me in my daily experiments. They are always there to listen to my report and give advice. They taught me practical techniques in detail test and revised my paperwork. My success in research results cannot be achieved without their effort.

Besides them, I must thank current and past members in Dr. Li's group, Ms. Lisa Cooney, Dr. Jianhan Lin, Mr. Kentu Lassiter, Ms. Damira Kanayeva, Mr. Jacob Lum, Dr. Min Li, Mr. Haibo Huang, Mr. Shichuang Liu, Ms. Yun Wang for their assistance and cooperation that made



this research successful. We worked together happily and become good friends in these two years.

I would also like to acknowledge Dr. Huaguang Lu and his group at the Animal Diagnostic Laboratory, Pennsylvania State University. He provided me with avian influenza diagnostic training during my research and continuously supplied us with virus samples that I needed in research.

Here I would also give thanks to Dr. Douglas Rhoads, the director of the Cell and Molecular Biology Program. He helped me in organizing my academic plan during the past two years.

I would appreciate the Center of Excellence for Poultry Science for providing the facility and equipment. Special thank also goes to Arkansas Bioscience Institute's grant to support this research.

Finally, I wish to thank my parents and my friends for their constant love, encouragement, and support over the years.

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## **CHAPTER 1 INTRODUCTION**



High-pathogenicity avian influenza (HPAI) causes rapid mortality in poultry, which often approaches 100% of incidences (Alexander, 2000). Recently, continuous outbreaks of HPAI have been reported in Asia and Europe (Horimoto and Kawaoka, 2005). AI virus H5N1 was first reported in 1997 in Hong Kong (Saw et al., 1997; Mounts et al., 1999).

The link between human and animal infection has raised concern among public health authorities and the scientific community about the prevalence and pandemic potential of AI virus. In the past several years, there has been a substantial increase in the number of AI outbreaks in poultry flocks and the number of countries reporting outbreaks continues to increase (Capua and Alexander, 2004; Zhao et al., 2005; Capua and Marangon, 2006). From 1999 to 2003, more than 50 million birds died or were eradicated after an outbreak of HPAI in the European Union (Capua and Alexander, 2004). In the US, a recent outbreak of low pathogenic AI (LPAI) in 2001 and 2002 resulted in the depopulation of over 4.5 million chickens and turkeys and is estimated to have cost the poultry industry approximately \$125 million (Dunn et al., 2003). There are 502 confirmed human cases and 298 deaths reported by WHO until July 2010 (WHO, 2010).

As a result, pathogenic AI virus H5 and H7 represent a potential danger not only to poultry but also to human health. Therefore, in addition to containment procedures, sensitive detection assays for early diagnosis are vital to lower the chances of spread and reduce the risk of development into an epidemic. Rapid and specific detection of avian influenza (AI) virus is becoming increasingly important and urgent in the face of concerns over the outbreaks of highly pathogenic AI H5N1 and the cases of human infection. Among AI subtypes, only the representatives of subtype H5 and H7 have been shown to exhibit highly pathogenic characteristics and to cause disastrous epidemic disease. The technology of diagnosing AI infections is available, such as viral culture, RT-PCR and ELISA methods, but many tests are too

complex and some can be performed only in biosafety level 3 facilities. Therefore, we proposed in the research to develop a DNA-aptamer based SPR biosensor coupled with magnetic bio-nanobeads separation for rapid, specific, sensitive, inexpensive and simultaneous detection of AI pathogenic virus H5 in less than 1 h in the field or at the patient's bedside.

## **CHAPTER 2 LITERATURE REVIEW**

## 2.1 Overview of Avian Influenza Virus

Avian influenza is an infection caused by avian influenza viruses. These influenza viruses infections occur naturally among birds. Wild birds worldwide carry the viruses in their intestines, seldom getting sick from them. However, contagious avian influenza can make some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them (CDC, 2007).

Influenza A (H5N1) virus, also called “H5N1 virus”, is an influenza A virus subtype that occurs mainly in birds, is highly contagious among birds, and can be deadly to them. H5N1 virus does not usually infect people, but infections with these viruses have occurred in humans. Most of these cases have resulted from people having direct or close contact with H5N1-infected poultry or H5N1-contaminated surfaces (WHO, 2007).

Avian Influenza Virus is 80-120 nm diameter single-strained, negative-sense ribonucleic acid (RNA) viruses with a segmented genome. It can be further classified into various subtypes on the basis of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) proteins (Murphy and Webster, 1996). So far there are sixteen HA (H1 through H16) and nine NA (N1 through N9) (Fouchier et al., 2005). Influenza virus specificity for the host is mediated by the viral surface glycoprotein hemagglutinin (HA), which binds to receptors containing glycans with terminal sialic acids. The molecular-recognition process leads to the host cell-virus adhesion stage (Paulson et al., 1985). The sialic acid receptor of various influenza virus strains differs in affinity to sialic acids terminally linked either in  $\alpha$  (2, 3) or  $\alpha$  (2, 6) position to the galactose (Gal) residues. Human influenza A virus preferentially recognize the  $\alpha$  (2, 6)-linkage, while avian virus have preference to the  $\alpha$  (2, 3)-linkage (Rogers and Paulson, 1983). Each monovalent sialic acid-binding site of HA is weak, with dissociation constants in the mill molar range, while the high virus-cell affinity is due to multivalency (Glick et al., 1991).

## 2.2 Current Diagnostic Techniques for Influenza Virus

Traditional diagnostic methods and rapid diagnostic methods frequently used in current detection for Influenza Virus were concluded in Table 2.1 and 2.2

**Table 2.1** Traditional Influenza Diagnostic Methods

(Centers for Disease Control and Prevention,

<http://www.cdc.gov/flu/professionals/diagnosis/rapidclin.htm>)

Procedure	Influenza Types Detected	Acceptable Specimens	Time for Results
Viral culture	A and B	Nasopharyngeal(NP) swab, throat swab, nasal wash, bronchial wash, nasal aspirate, sputum	3-10 days
Immunofluorescence DFA Antibody Staining	A and B	NP swab, nasal wash, bronchial wash, nasal aspirate, sputum	2-4 h
RT-PCR	A and B	NP swab, throat swab, nasal wash, bronchial wash, nasal aspirate, sputum	2-4 h
Serology	A and B	paired acute and convalescent serum samples	2 weeks or more
Enzyme Immuno Assay (EIA)	A and B	NP swab, throat swab, nasal wash, bronchial wash	2 h

**Table 2.2** Rapid Diagnostic Methods for Influenza Disease

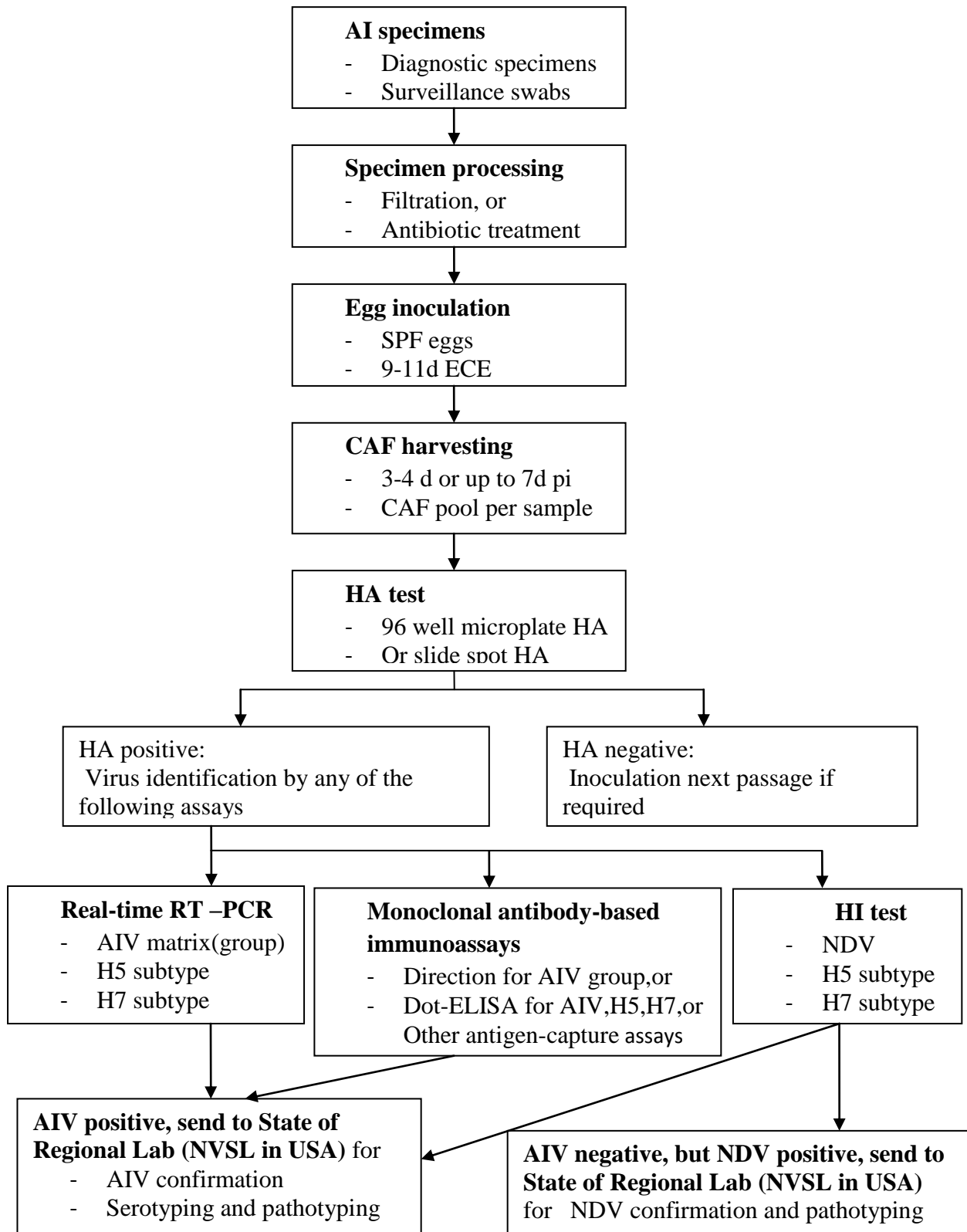
(Centers for Disease Control and Prevention,

<http://www.cdc.gov/flu/professionals/diagnosis/rapidclin.htm>)

Procedure	Influenza Types Detected	Acceptable Specimens	Time for Results
3M™ Rapid Detection Flu A+B Test(3M)	A and B	NP swab/aspirate; Nasal wash/aspirate	15 min
Directigen EZ Flu A+B (Becton-Dickinson)	A and B	NP wash/aspirate/swab; lower nasal swab; throat swab; bronchioalveolar lavage	less than 15 min
BinaxNOW Influenza A&B (Inverness)	A and B	Nasal wash/aspirate, NP swab	less than 15 min
OSOM® Influenza A&B (Genzyme)	A and B	Nasal swab	less than 15 min
QuickVue Influenza Test (Quidel)	A and B	NP swab, nasal wash, nasal aspirate	less than 15 min
QuickVue Influenza A+B Test (Quidel)	A and B	NP swab, nasal wash, nasal aspirate	less than 15 min
SAS FluAlert (SA Scientific)	A and B	Nasal wash/aspirate	less than 15 min
TRU FLU (MeridianBioscience)	A and B	Nasal wash/swab, NP aspirate/swab	15 min
XPECT Flu A&B (Remel)	A and B	Nasal wash, NP swab, throat swab	less than 15 min

The ideal diagnostic method would have high sensitivity and specificity for the identification of influenza, would be relatively simple to administer, and would be rapid and inexpensive. The timeliness of results is critical because, in order to be effective, the newer therapies (as well as the more traditional antiviral therapies for influenza A must be started within 48 h after onset of symptoms. As test accuracy improves or cost falls, the argument for diagnostic testing is enhanced. Regardless of the sensitivity and specificity of each test, the positive predictive value of any test will improve when the prevalence of influenza in the community is high. On the other hand, as the likelihood of influenza illness increases (such as in major outbreaks), the effectiveness and tolerability of treatment improve, or the cost of treatment decreases, an advantage is realized for empiric treatment based on clinical characteristics alone (Cram et al., 1999).

The golden standard virus detection method is based on virus isolation culture with rapid immunoassay procedure, such as direct immunofluorescent assay or membrane enzyme-linked immunosorbent assay, followed by characterization with serological or molecular biological methods (Stamboulia et al., 2000; Boon et al., 2001). However, they are time consuming, poor in specificity, and low in sensitivity. On the other hand, RNA based methods, especially RT-PCR, has gained tremendous ground as an effective tool for viral detection, typing and subtyping (Zhang and Evans, 1991). But RNA based methods are still typically time-consuming to conduct since the protocol requires a series of RNA isolation, concentration, and gel electrophoresis. Fig.2.1 shows golden standard virus detection process for Influenza Virus.



**Fig.2.1.** Flow chart of isolation and identification of Avian Influenza Virus



### 2.3 Biosensors for Influenza Virus Detection

Rapid detection techniques should provide reliable, real time, on-field, user-friendly, and inexpensive detection with improved or equivalent sensitivity, specificity and reproducibility of culture-based tests (Alocilja and Radke, 2003). Current interest has focused in developing new influenza sensors for quick and reliable testing for influenza with minimum sample handling and laboratory skill requirement. One of the strategies is to develop single step direct sensing methods that eliminate separation, incubation or use of any signal-reporting agents. According to Lazcka et al. (2007), biosensor technology is the fastest growing area in rapid pathogen detection. In recent years, various biosensors have been studied as alternatives to conventional methods for detection of influenza viruses. The commonly used biological recognition elements in biosensor platforms are antibodies and nucleic acid probes. Compared to conventional diagnostic technologies, the biosensor has the following advantages:

- 1) Fast: biosensor has the potential to detect AI virus in less than 1 hr.
- 2) Reusable: sensing surface throughout a number of assay cycles can be reused.
- 3) Minimal requirement on sample handing and laboratory skill.
- 4) Quantifiable: electrical signal can be further processed to get extensive information.

Non-labeling techniques such as surface plasmon resonance (SPR) and quartz-crystal microbalance (QCM) have shown high promise in sensor research and gained momentum in detection of viral samples. Detection limits of QCM and SPR are both around the sub-nanogram region. Because the molecular weight of influenza A/B virus is about  $2.5 \times 10^6$ , the detectable number of virions is considered to be around  $10^6$ . Another promising method is colorimetric sensors with functional polymers, which can provide a litmus test type of detection.

QCM has shown high promise in sensor research and gained momentum in detection of influenza virus even they have limited success in specificity (Schofield and Dimmock, 1996; Kortt et al., 1999; Sato et al., 1999).

Surface Plasmon Resonance (SPR) is a direct optical-sensing technique (Homola, 2003). Its label-free detection scheme together with very high sensitivity offers enormous opportunity and flexibility to biosensing assay development (Cooper, 2003). The principle of SPR has been the subject of several reviews (Sambles et al., 1991). Judging from the increasing number of publications, it is clear that surface plasmon resonance has techniques for detection of various biochemical analytes (Mullett et al., 2000). Surface plasmon resonance has been used for detection of influenza virus and the study of interactions that involve viral proteins and receptors. The first use of SPR in influenza virus detection was reported by Schofield and Dimmock (1996). The sensor chip was coated with carboxylated dextran polymer matrix, on which monoclonal antibody HC10 for capture of influenza virus was coupled. Influenza virus was injected into the flow system, and the binding affinity with the surface antibody was monitored. The dissociation rate constant and association rate constant were found similar to those obtained with an affinity ELISA. The study demonstrates the feasibility of affinity measurements and detection for large virus particles by SPR. This study was followed by Critchley and Dimmock (2004) who studied the binding of influenza A virus to a neomembrane composed of bovine brain lipids that contains sialoglycolipids. The detection of plant viruses has also been successfully demonstrated by SPR (Boltovets et al., 2002). For the binding affinity area, Takemoto et al. (1996) developed a binding assay to study the interaction between influenza hemagglutinin and its cell surface receptor. Low-pH-induced BHA rosettes bind specifically to the fetuin-derivitized sensor surface, but not to an asialofetuin-derivitized sensor surface. The association rate, dissociation rate, and

dissociation constant for the multivalent interaction between BHA rosettes and the fetuin-derivitized sensor surface were measured, enabling quantitation of the multivalent binding between BHA rosettes and the fetuin-derivitized sensor surface. Kortt et al. (1999) measured the binding of the Fab fragment of monoclonal antibody NC10 to influenza virus N9 neuraminidase, isolated from tern and whale. A contribution of mass transport to the kinetic constants was demonstrated at higher surface densities and low flow rates, and was minimized at low ligand densities and relatively high flow rates (up to 100 ml/min). Nice et al. (1996) examined the interactions between two monoclonal antibodies and their corresponding Fab' fragments with a synthetic peptide. The peptide corresponds to the C-terminal 23 residues of the HA1 chain of influenza virus hemagglutinin. The IgGs were found to have equilibrium association constants ( $K_A$ ) 10 to 20-fold higher than the corresponding Fab' fragments.

There is much interest in developing new SPR influenza biosensor for quick and reliable testing for influenza. SPR biosensor has the advantage of simplifying the analytical procedure by reducing need for labeling replaced by detecting the analyte itself. Very limited papers have been published about SPR influenza biosensor, which were based on antibody-antigen binding (Schofield and Dimmock, 1996; Kortt et al., 1999) or sialic acid binding (Critchley and Dimmock, 2004), and so far, there is no report about SPR biosensor combining with magnetic bio-nanobeads separation and used for in-field swab sample AIV test of swab samples.

## **2.4 Aptamers**

There is new emerging area of aptamers' application in detection. Aptamers, whose name is derived from the Latin word aptus meaning "to fit", are oligonucleotides (DNA or RNA) that can bind with high affinity and specificity to a wide range of target molecules, such as drugs,

proteins or other organic or inorganic molecules (Jayasena, 1999; Patel and Suri, 2000; Clark and Remcho, 2002; Luzzi et al., 2003; You et al., 2003). Aptamers are generated by an in vitro selection process called SELEX (systematic evolution of ligands by exponential enrichment). SELEX was first reported in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990) and it has permitted the identification of unique RNA/DNA molecules, from very large populations of random sequence oligomers (DNA or RNA libraries). Selections are frequently carried out with RNA pools due to the known ability of RNAs to fold into complex structures which can be a source of diversity of RNA function, but single-stranded DNA pools can also yield aptamers (Marshall et al., 2000). Single-stranded DNAs are also known to fold in vitro into structures containing stem-loop, internal loops, etc., even if less stable than the corresponding RNA structures (Harada et al., 1994).

Aptamers show a very high affinity for their targets, with dissociation constants typically from the micromolar to low picomolar range, better than those of some monoclonal antibodies (Jenison et al., 1994). Aptamers have also been selected with a high binding specificity, as demonstrated by an anti-theophyllin aptamer that displays a 10,000-fold discrimination against caffeine (difference of only a methyl group) (Jenison et al., 1994). The large number of possible oligonucleotide sequences and their molecular diversity allow the isolation of aptamers with affinity for a large variety of molecules. High-affinity aptamers have been selected to bind an hundred different targets such as organic dyes (Ellington et al., 1992), amino acids (Famulok et al., 1992; Geiger et al., 1996), antibiotics (Schurer et al., 2001; Tereshko et al., 2003; Zimmerman and Maher, 2002), peptides (Baskerville et al., 1999), proteins (Klug et al., 1999).

In comparison to antibodies, aptamer receptors have a number of advantages that make them promising in analytical and diagnostic applications. Table 2.3 shows properties comparison between aptamers and antibodies.

**Table 2.3** Physical Properties of Aptamers versus Antibodies Applied in Diagnostics

Aptamers	Antibodies
In vitro selection is chemical process and target any protein under a variety of conditions	generation requires a biological system and is limited to physiologic conditions
Unlimited shelf life	Limited shelf life
Temperature stable	Temperature sensitive, irreversible denaturation
Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch (polyclonal antibody)
chemical modifications to molecule for diverse functions	Limited modifications of molecule
Binding affinity in low nanomolar to picomolar range	Binding affinity in low nanomolar to picomolar range

The aptamer selection process can be manipulated to obtain aptamers that bind a specific region of the target and with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using polyclonal antibodies and, by chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules. Finally, because of their simple structure, sensor layers based on aptamers can be regenerated more easily than antibody-based layers, are more resistant to denaturation and have a much longer shelf life (Mok and Li, 2008).

Moreover, the selections process itself, with the amplification step, gives some advantages to aptamers with respect to other “non-natural” receptors, such as oligopeptides, which cannot be amplified during their selection procedure.

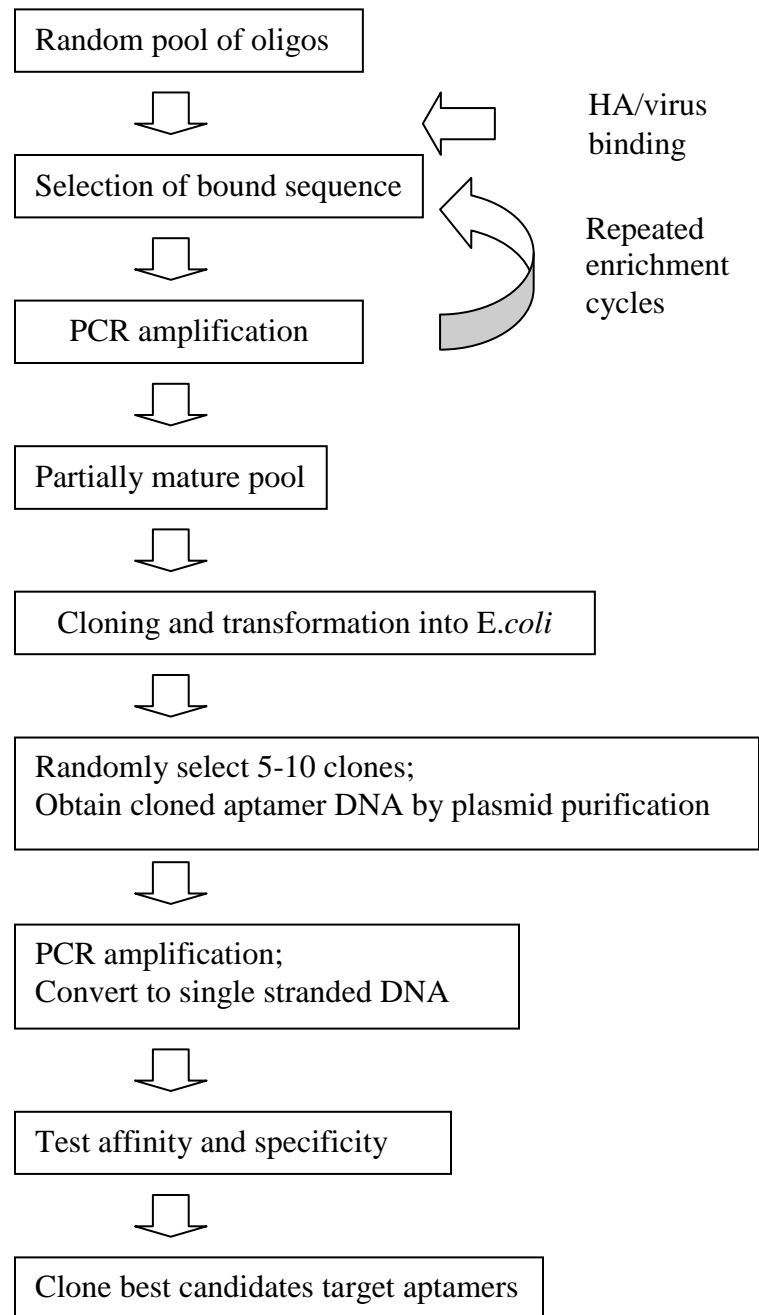
The primary limitation on the use of aptamers (mainly RNA aptamers) as recognition elements has been their nuclease sensitivity which is very critical for their use in *ex vivo* and *in vivo* applications (Famulok et al., 2000). However, it has been shown that the stability of such molecules can be improved by chemical modification of the ribose ring at the 2-position (Pieken et al., 1991). Because the nucleases that are most abundant in biological fluids are the pyrimidine-specific nucleases, introduction of specific modifications at the 2- position of pyrimidine nucleotides (2-amino and 2-fluoro functional groups) protects an RNA oligonucleotide from degradation, increasing the half-life up to 15 h (Heidenreich and Eckstein, 1992). Because 2-amino and 2-fluoro CTP and UTP can be incorporated into *in vitro* transcribed RNA, these modifications can be introduced into the combinatorial library. In this way, it has been possible to select aptamers with enhanced stability in biological fluids from libraries containing modified pyrimidines with 2-amino and 2-fluoro (Kusser, 2000). Due to all these characteristics, aptamers have been used in numerous investigations, as diagnostic tools (Brody and Gold, 2000) and for the development of new drugs. Moreover, aptamers have been recently used in analytical chemistry applications, as immobilized ligands or in homogeneous assays.

## **2.5 SELEX Process**

Aptamers were discovered by the development of the SELEX (systematic evolution of ligands by exponential enrichment) process (Ellington et al., 1990; Tuerk et al., 1990) which is a technique for screening large libraries of oligonucleotides by an iterative process of *in vitro* selection and amplification.

The SELEX process (Fig. 2.2) starts with the synthesis of a single-stranded library of oligonucleotides; each oligonucleotide comprises a central region of random sequence flanked

by a 5' and a 3' region of defined sequence. Each member in a library is a linear oligomer of a unique sequence.



**Fig. 2.2** Systematic evolution of ligands and exponential enrichment (SELEX) and DNA-aptamers selection scheme

The complexity of the library is dependent on the number of randomised nucleotides positions. Normally, the starting round contains  $10^{13}$  to  $10^{15}$  individual sequences, a very large number that permits to generate a high probability of selecting an aptamer specific for the target of interest.

The chemically synthesised single-stranded DNA pool is amplified by polymerase chain reaction (PCR) in order to generate a double-stranded DNA pool. This is then either transcribed (for RNA selection) or strand separated (for single-stranded DNA selection) to convert it into a suitable form for selection (Wilson and Szostak, 1999).

Three dimensional structures of these RNA/ssDNA sequences will result in a combination of Watson–Crick base-pairing and intramolecular interactions. To isolate aptamers to a given target molecule, the starting library of nucleic acids is incubated with the molecule of interest. The rare nucleic acid ligands, that adopt conformations that allow them to bind to the specific target, are partitioned by filtration through nitrocellulose (for protein target) or by affinity chromatography (normally for small molecules target). The population of molecules that bound the target is eluted and amplified by RT-PCR (for RNA libraries) or PCR (for DNA libraries) to have a new lower complexity pool enriched in target binding species that, in the case of RNA libraries, will be in vitro transcribed and used for the next selection/ amplification cycle. The enrichment of the high-affinity sequences at the expense of the low-affinity binders requires several iterations of the selection process carried out under increasing stringent conditions. A negative selection step is frequently employed by passing the nucleic acid pool over a cellulose filter in the absence of the target; this step eliminates the aptamers that bind the filters in a target-independent manner (Luzi et al., 2003). The number of cycles required is dependent on the stringency imposed on each round as well as on the affinity of interaction between the target and



the aptamers. In general, around 8–15 cycles of affinity selection and amplification are needed before selecting an oligonucleotide population that is dominated by those sequences which bind the target best. Cloning and sequencing of the selected clones will reveal the sequence and the structure of the specific selected ligands.

Once obtained this information, the aptamer can be generated by chemical synthesis. Its binding activity can be confirmed by different methods to validate its affinity and specificity for the target.

Since most in vitro selection experiments take from weeks to months to be completed as described above, the SELEX process has been recently automated (Cox and Ellington, 2001; Cox et al., 2002a,b). A novel in vitro selection methodology has been also proposed by Golden et al. (2000). With this new photochemical SELEX (PhotoSELEX) method, modified ssDNA aptamers capable of photocross-linking the target molecule (human basic fibroblast growth factor (bFGF)) have been identified. The method is based on the incorporation of a modified nucleotide activated by absorption of light, in place of a native base in either RNA or in ssDNA-randomised oligonucleotide libraries. The aptamers selected with this method have the ability to form a photo-induced covalent bond with the target molecule.

## **2.6 Aptamer-based Biosensors**

Aptamer detection applications include aptamer-based microarrays (Collett et al., 2005), quantum-dot aptamer complexes (Levy et al., 2005; Hansen et al., 2006), aptamer-functionalized gold nanoparticles (Huang et al., 2005; Kouassi et al., 2007), and aptamer-based biosensors (aptasensors) (Deisingh, 2006; Willner et al., 2007; Fischer et al., 2007; Lee et al., 2008). Electrical and optical aptasensor platforms have been reviewed previously, including aptasensor

applications in biosecurity and clinical diagnostics (O'Sullivan, 2002; Li et al., 2008; Song et al., 2008). The application of aptamers as biocomponents in biosensors offers advantages over classical affinity sensing methods mainly based on antibodies. These advantages include the possibility to easily regenerate the function of immobilized aptamers, their homogeneous preparation and the possibility of using different detection methods due to easy labeling (Jayasena, 1999; O'Sullivan, 2002; Luzi et al., 2003; You et al., 2003).

Aptamers have been used as bio-recognition element in optical sensors, both labelled (fluorescence-based methods) or in systems not requiring labels (surface plasmon resonance). Fluorophores remain one of the most prevalent signal transducers to be incorporated into aptamer biosensors, primarily thanks to the easy conjugation of fluorophores onto nucleic acid sequences and the convenience of detecting the fluorescence signals. The design and characteristics of these sensors are extensively discussed in a previous review by Nutiu and Li (2005). Immobilized optical sensors can be represented by unlabelled surface plasmon resonance biosensor. When light strikes the surface of a metal, especially silver and gold, at a particular angle, it can excite electrons on the surface, causing them to resonate and be propagated along the metal. This phenomenon, known as surface plasmon resonance (SPR), may be used to generate a signal to communicate target detection. The signal transducer used in SPR-based aptamer biosensors is generally gold covered disks coated with streptavidin. The presence of streptavidin, a biotin binding protein, enables the immobilization of biotinylated aptamers. When these chips are exposed to their targets, the interaction between the aptamers and their ligands would alter the local refractive index, and in turn change the surface plasmon resonance in response to excitation by the incident light. This fluctuation may be detected by monitoring the change in SPR angle, which is defined as the angle at which the reflected light reaches a

minimum intensity, following ligand binding. This system has been previously applied by Tombelli and colleagues for the detection of the HIV-1 Tat (trans-activator of transcription) protein (Tombelli et al., 2005) and by Tang and colleagues, who developed a sensor decorated with antithrombin DNA aptamers (Tang et al., 2007).

The detection of changes in SPR and surface plasmon coupling does not always depend on sophisticated instruments as in the aforementioned sensors. In the case of gold nanoparticles (AuNPs), changes in SPR associated with the transition from the colloidal dispersion state to the aggregation state contribute to a red to violet color change that is observable with the naked eye (Okamoto et al., 2003). This unique property of AuNPs was recognized by mankind as early as the Middle Ages, when these particles were used in tainting stain glass and embellishing decorations with vibrant red and blue colors although the exact mechanism behind the color change was a mystery (Rao et al., 2000). In modern times, these particles are actively studied by the scientific community and new applications for them are constantly emerging.

The development of oligonucleotide-modified AuNPs as probes for the selective and sensitive detection of complementary sequences by Mirkin and his coworkers back in 1997 painted the possibility of chemically conjugating these particles with nucleic acids and exploiting their unique distance-dependent optical properties for signaling (Elghanian et al., 1997). Making use of oligonucleotide hybridization and AuNPs, Liu and Lu designed ATP and cocaine aptamer biosensors that use AuNPs as signal transducers (Liu et al., 2006). In this system, the aptamers are connected to a linker sequence. The nanoparticles are either functionalized with a sequence that is complementary to the 5' end of the linker or one that is complementary to the 3' end of the linker and part of the aptamer. When the target is absent, the linker and aptamer bind to both oligo 1 and oligo 2, thereby crosslinking the AuNPs and forming aggregates that appear violet

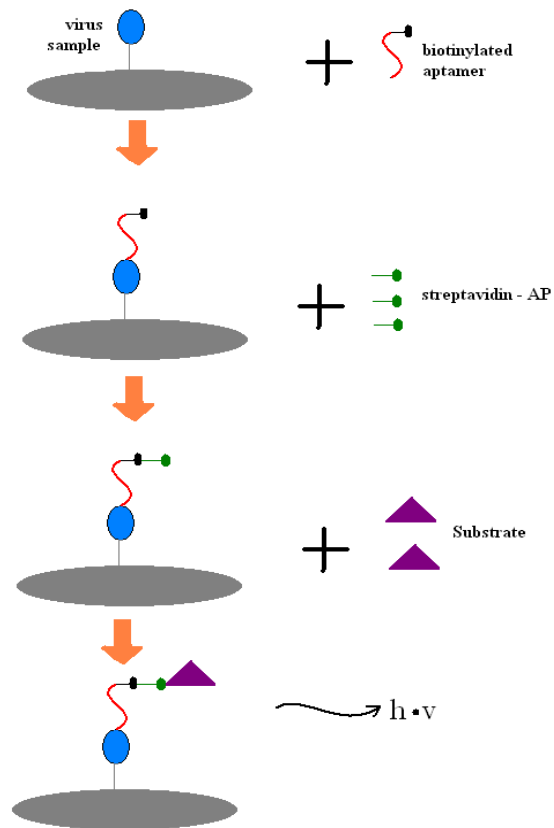
when suspended in solution. To accommodate their targets, the aptamers adopt folded structures, which displace oligo 2 while the linkers are still bound to oligo 1. This leads to the breakdown of the aggregates and a violet-to-red color change in solution.

## **2.7 Dot ELISA**

Dot-ELISA (Enzyme Linked Immunosorbent Assay) is a commonly used immunological tool in diagnostic laboratories. Sandwich Dot-ELISA would be used in this study. Here viruses were immobilized onto a solid support and streptavidin-AP conjugate was linked to an enzyme. Biotin-labeled aptamer in the test sample first reacted with the immobilized virus and then with the second enzyme-linked conjugate. The amount of enzyme linked conjugate bound was assayed by incubating the strip with an appropriate chromogenic substrate, which was converted to a colored, insoluble product (Fig. 2.3).

The latter precipitates onto the strip in the area of enzyme activity, hence the name Dot-ELISA. The enzyme activity is indicated by the intensity of the purple spot, which is directly proportional to the antigen concentration.

Dot ELISA was a good test kit because of its specificity and had high sensitivity when compared to standard immunoblotting assay. According to references Pappas et al. (1983) and Lu (2003), The sensitivity and specificity of the Dot ELISA assay are better than standard micro-ELISA. Meanwhile dot-ELISA is preferred compare to microwell ELISA test because it is simple to perform and needs less test time.



**Fig. 2.3** Flow chart of sandwich Dot ELISA method in which biotin-labeled aptamers were linked to streptavidin-AP conjugate and viruses

## **CHAPTER 3 OBJECTIVES**

Pathogenic AI virus H5 and H7 caused rapid mortality in poultry and threaten to human health. Research in diagnostics of AI virus lasted for a long time. But limited reports for rapid detection assays are available; especially little research is focused on aptamer based assays. Considering aptamers in terms of good affinity and advantage of better thermal stability over antibodies, this study was aimed to selecte aptamers for detection of H5N1 virus.

In this study, SELEX (selective evolution of ligands by exponential enrichment) was designed to select DNA-aptamers, which could specifically recognize H5N1 AI virus. Then the selected aptamers would be characterized for their specificity and affinity using Dot ELISA and SPR test. Upon evaluation, these ssDNA aptamer would be used as recognition element of assays for rapid detection of AI virus H5N1.

## **CHAPTER 4 MATERIALS AND METHODS**



#### **4.1 Virus and Plasmid**

The influenza viruses used in the study were inactivated AIV H5N2, H5N3, H5N9, H1N1, H2N2, H7N2, H9N2 strain from Animal Diagnostic Laboratory, Pennsylvania State University and inactivated AIV H5N1 sample (Scotland 59 H5N1) from USDA-APHIS National Veterinary Services Laboratories (NVSL, Ames, IA). The pGEM T easy vector was obtained from Promega (Madison, WI).

#### **4.2 DNA library and Primers**

A pool of DNA oligonucleotides containing a region of randomized nucleotides (74 nt) flanked by constant sequences for binding of primers for PCR. The DNA oligonucleotides (5'-CCGGAATTCCTAATACGACTC-N74-TATTGAAAACGCGGCCGCGG-3') was synthesized by IDT (integrated DNA Technologies). The forward primer biotinylated DL-F (5'-bio-CCGGAATTCCTAATACGACTC-3') and reverse primer phosphorylated DL-R (5'-phosphate-CCGCGGCCGCGTTTTCAATA-3') were used to amplify the double strand aptamers candidates and ssDNA which were applied for the next selection cycle.

#### **4.3 Selection of DNA Aptamers Binding to H5N1**

Selection of DNA aptamers with a high affinity for H5N1 viruses was performed as follows. The DNA library mixed with H5N1 viruses. Then, the mixture passed through a filter. Few nucleic acids that reveal affinity and specificity towards the H5N1 stayed on the filter, while most of the library ingredients were washed away. The DNA-aptamer candidates were then eluted from the filter, and followed by PCR amplification. Subsequent selection cycles were

similar to the earlier ones with the exception that the stringency of selection would be increased to promote competition between binding species. Therefore, as the cycles progress, the molar ratio of HA to DNA (including pool DNA, nonspecific DNA) was increased from 1:20 to 1:500 (Table 4.1). after 4 cycles, since NA proteins are unavailable, H5N1 virus was incubated as a substitute of NA proteins with aptamers pool in following nine cycles. 13 repeated separation-amplification cycles yielded higher affinity and specificity DNA-aptamers.

**Table 4.1** Aptamers Binding with H5N1 Virus Mixture Ratios

Cycle	HA protein ( $\mu\text{M}$ )	ssDNA Pool ( $\mu\text{M}$ )
1	0.5	5.0
2	0.25	5.0
3	0.125	5.0
4	0.063	5.0
	H5N1 ( $\mu\text{l}$ )	ssDNA Pool ( $\mu\text{M}$ )
5	30	0.7
6	25	1.7
7	20	1
8	15	0.8
9	10	1.1
10	5	0.85
11	2	0.9
12	1	0.8
13	0.5	0.8

### 4.3.1 SELEX

Reagents used in SELEX included: binding buffer (50 mM Tris-HCl, 2.5 mM NaCl, 5 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol, PH 7.5), washing buffer (the same as binding buffer), elution buffer (0.4 M Sodium acetate, 5mM EDTA, 7M Urea, PH 5.5).

The detail protocol for SELEX procedure was described as follows.

1. 50  $\mu\text{l}$  ssDNA, 1.5  $\mu\text{l}$  glycogen and 50  $\mu\text{l}$  Ammonium acetate (7.5 M) was dissolved in 0.25 ml ethanol (100%). The solution was incubated for 30 min at  $-80\text{ }^\circ\text{C}$  and then centrifuged

under 13000 rpm at 4 °C for 30 min. The pellet was washed with 75% ethanol solution (-20 °C)(100 µl) twice and finally resolved in 100 µl binding buffer solution. Concentration of the ssDNA solution was measured

2. 100 µl ssDNA solution was denatured at 95 °C for 10 min and cooled at room temperature for 30 min.
3. Nitrocellulose acetate membrane (HAWP filter, 0.45 µl) was soaked in binding buffer solution for 1 min, and then the membrane was put into the filter holder. In order to eliminate ssDNA that bound to the membrane, the denatured ssDNA solution was passed three times prior to the selection cycle through pre-wetted membrane in “pop-top” filter holders (see Appendix A).
4. 100 µl denatured ssDNA solution and HA protein or H5N1 (128 HA unit) (according to mixture ratio in Table 4.1) were added into binding buffer to reach total solution volume of 200 µl. Solution was incubated at 15 rpm on a variable speed rotator for 1 h at room temperature.
5. 1 ml washing buffer (the same as binding buffer) was then passed through the membrane.
6. ssDNA retained on the filter were eluted twice with 200 µl of elution buffer at 70-80 °C over the course of 5 min.
7. 400 µl eluted ssDNA solution was separated into 2 tubes. 150 µl H<sub>2</sub>O, 4.5 µl glycogen, 200 µl Ammonium acetate (7.5 M) and 1 ml ethanol (100%) were added into each tube and 2 tubes of solution were incubated for 30 min at -80 °C. Then solutions were centrifuged under 13000 rpm at 4 °C for 30 min. Pellets were washed with 75% ethanol solution(-20 °C)(100 µl) 2 times and resolved in 50 µl pure water. Finally they are ready for PCR.

### 4.3.2 Amplification and $\lambda$ Digest of Aptamers Pool

#### 4.3.2.1 PCR

PCR was carried out in the following reaction mixture (Table 4.2)

**Table 4.2** NEB TAQ System for PCR Aptamers Pool

Componets of PCR reaction	Volume ( $\mu$ l)
DNA template	1
10 $\times$ thermol buffer	5
dNTP	4
Forward primer DL-F-bio	1
Reverse primer DL-R-phos	1
Taq Polymerase	0.5
dh <sub>2</sub> 0	37.5

Amplification program was set as: 95  $^{\circ}$ C for 5 min followed by 30 cycles of 94  $^{\circ}$ C for 45 s, 64  $^{\circ}$ C for 45 s and 72  $^{\circ}$ C for 45 s and lastly followed by 72  $^{\circ}$ C 10 min.

#### 4.3.2.2 $\lambda$ Digest

For the purpose of obtaining ssDNA aptamers pool,  $\lambda$  digest was performed at 37  $^{\circ}$ C for 1 hr, following 75  $^{\circ}$ C for 10 min (Table 4.3).

**Table 4.3**  $\lambda$  Digest Reaction Mixture for Converting dsDNA to ssDNA Aptamers

	Sample ( $\mu$ l)	Blank ( $\mu$ l)
DNA template	40	0
$\lambda$ expo	1	1
10 $\times$ buffer	5	5
Deionized water	4	44
total	50	50

## 4.4 Cloning and Sequencing

### 4.4.1 Ligation

pGEM-T Easy Vectors system was used in the ligation reaction. First, the pGEM-T Easy vector and control insert DNA tubes were briefly centrifuged to collect the contents at the bottom of the tubes. Then three ligation reactions were set up as described below (Table 4.4).

**Table 4.4** Ligation Reactions Mixture using pGEM-T Easy Vectors System

	Aptamers ( $\mu$ l)	Positive Control ( $\mu$ l)	Background Control ( $\mu$ l)
2 $\times$ Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
pGEM T easy Vector (50 ng)	1	1	1
Target DNA product	1	0	0
Control Insert DNA	0	2	0
T4 DNA Ligase (3 weiss units/ $\mu$ l)	1	1	1
Deionized water( $\mu$ l)	2	1	3

### 4.4.2 Transformation

Transformation aimed to sequence aptamers was carried out in the following steps.

1. Prepared two LB/ampicillin/IPTG/X-Gal (Sigma/Invitrogen) plates for each ligation reaction. Equilibrated the plates to room temperature.
2. Centrifuged the tubes containing the ligation reactions to collect the contents at the bottom. Added 2  $\mu$ l of each ligation reaction to a 1.5 ml sterile microcentrifuge tube on ice.
3. Removed tubes of frozen JM109 High Efficiency Competent Cells (Promega) from storage and placed in an ice bath until just thawed (about 5 min). Mixed the cells by gently flicking the tube.
4. Carefully transferred 50  $\mu$ l of cells into each tube prepared in Step 2.

5. Gently flicked the tubes to mix and place them on ice for 20 min.
6. Heat-shocked the cells for 45–50 s in a water bath at exactly 42 °C (do not shake).
7. Immediately returned the tubes to ice for 2 min.
8. Added 950 µl room-temperature SOC medium (Invitrogen) to the tubes containing cells transformed with ligation reactions.
9. Incubated for 1.5 h at 37 °C with shaking (~150 rpm).
10. Plated 100 µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. To obtain a higher number of colonies, the cells were then pelleted by centrifugation at  $1,000 \times g$  for 10 min, resuspended in 200 µl of SOC medium, and 100 µl plated on each of two plates.
11. Incubated the plates overnight (16–24 h) at 37 °C.

#### **4.4.3 Colony PCR**

Each colony was suspended in 20 µl PBS. Colony PCR was performed as follows (Tables 4.5). PCR program was set as follows: diluted colonies were heated at 94 °C for 10 min and then reacted with other PCR system components 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 3 min and lastly followed by 72 °C 10 min.

**Table 4.5** Colony PCR Mixture for Selected Aptamers' Amplification and Sequencing

Components	Volume ( $\mu$ l)
Diluted Colony	1-2
10 $\times$ buffer	5
MgCl <sub>2</sub> (25 mM)	4
dNTP (2.5 mM)	4
Forward primer DL-F (20 $\mu$ M)	1
Reverse primer DL-R (20 $\mu$ M)	1
Taq polymerase	0.5
dH <sub>2</sub> O	32.5
Total	50

#### 4.4.4 Sequencing

Sequencing was performed by ABI 3130xe analyzer (DNA Resource Center, University of Arkansas, Fayetteville).

### 4.5 Evaluation of DNA Aptamers

#### 4.5.1 Dot ELISA Test

Dot ELISA test was carried out first to do preliminary evaluation of DNA aptamers as follows.

1. Used clean scissors to prepare test strips of nitrocellulose membrane, 60 mm  $\times$  12 mm for 5 specimens. Vinyl gloves must be worn at all times when handling the nitrocellulose membrane.
2. Added 5  $\mu$ l test viruses (H5N1, H5N2, H5N3, H7N2, H9N2) on strips and allowed them to air dry, marked the upper side of membrane with pencil.

3. Transferred the strips to a clean Petri dish and laid out one by one. Strips with different test specimens could be put together but should not be overlapped.
4. Added a blocking solution to the strips (pour solution into dish and cover every strip) and allowed strips to react for 15-30 min. After the reaction, removed the strips and laid out on chromatography paper to air dry.
5. Transferred the strips into a new Petri dish and applied aptamers in an appropriate dilution (about 1:3, 50  $\mu$ l ssDNA aptamers +100  $\mu$ l blocking buffer) to the strips. Covered the entire strip surface with diluted aptamers (do not need pour, only added 150  $\mu$ l on every strip) and allowed this reaction to go for 45 min. kept them float on a supporter in water bath to avoid drying during process.
6. Strips were then washed with a wash buffer three times and each wash required a 5 min soak period. After washing, the washed strips were removed to allow them to air dry.
7. Transferred the strips into another new petri dish and added 200  $\mu$ l streptavidin-Alkaline Phosphatase conjugate at 1:500 dilution (diluted with blocking buffer), allowing this reaction to go for 25 min.
8. Washed the strips and air dry.
9. Transferred the strips into another new Petri dish and applied BCIP/NBT substrate to the strips. Maintained the strips and substrate in the dark to allow for color development, a purple color was evident within 5 min. (do not lasted too long time avoid of strong background).
10. Stopped the color development by immersing the strips in water for 10-20 s.
11. Allowed the strips to air dry in the dark. The strips could be stored in sealed plastic and kept in the dark to prevent the color from fading.



## **4.5.2 Dot Blot Test**

### **4.5.2.1 Dot Blot Test Materials**

In Dot Blot test, Avian Influenza Virus H5N1 (NVSL, Awes, IA) and H5N2 (ADL, state college, PA) were prepared as 1:100 dilution of stock sample. Biotin-ssDNA aptamers was 1:1 diluted w/blocking buffer. Streptavidin-HRP conjugated (Perkin Elmar, Boston MA) was 1:1250 diluted w/ blocking buffer. Blocking buffer was made of 2.5% (w/v) Casein, 0.15M NaCl, 0.01 M Tris and 0.02% Thimerosal; Adjust pH to 7.6 with NaOH.(reagents from VWR, Randor PA) Transfer buffer was made of 0.015 M Tris,0.12M Glycine, 20%(v/v) methanol(pH=8.3). (reagents from SIGMA, St. Louis MO) Substrate was Pierce Supersignal West Dura Extended Duration Substrate from Thermo Scientific, Rockford, IL.

### **4.5.2.2 Dot Blot Test Protocol**

Dot Blot test was conducted as following steps:

1. Soaked PVDF membrane in 100% methanol for 2 min.
2. Incubated membrane in transfer buffer for 10 min to wet.
3. Put PVDF in dot blot system and sealed system with vacuum.
4. Loaded samples in wells of plate.
5. Run vacuum on samples for 10- 20 min.
6. Placed blot in blocking buffer for 1h on shaker.
7. Incubated overnight in aptamers solution.
8. Washed 3× for 5 min each with blocking buffer.
9. Added streptavidin-HRP conjugated and incubated for 90 min.
10. Washed 3× for 5 min each with blocking buffer.
11. Added substrate (mix 1:1) in sealed bag and incubated for 5 min.

12. Removed substrate and placed blot in a plastic sheet protector.
13. Took pictures with chemiluminescence using the CCD camera.

### **4.5.3 Characterization of Selected Aptamers by Surface Plasmon Resonance**

#### **4.5.3.1 SPR Test Instruments and Materials**

For all experiments, the SPR instrument SensiQ (ICx Nomadics, Oklahoma City, Ok) and neutravidin modified sensor chips (BioCap) were used. All experiments were conducted at 25 °C with a flow rate of 10  $\mu$ l/min. The running buffer used for the experiments was PBS (10mM, pH 7.4). 10 mM NaOH (40  $\mu$ l) were used for regeneration to remove the bound target protein HA (Hemagglutinin) or AIV H5N1. The full-length glycosylated recombinant HA protein of the subtype H5N1 with concentration of 524  $\mu$ g/mL was purchased from Protein Science Corporation (Meriden, CT). The protein was produced in insect cells using the baculovirus expression vector system and purified to >90% purity under conditions that preserve its biological activity and tertiary structure. Inactivated AIV H5N1 sample (Scotland 59 H5N1) was obtained from USDA-APHIS National Veterinary Services Laboratories (NVSL, Ames, IA). Selected aptamers O16 was used in the SPR test.

#### **4.5.3.2 SPR Test Methods**

Immobilization of the selected aptamer on SPR chip surface was carried out by using the biotin-neutravidin method. The SPR chip was pre-coated with neutravidin, and then biotin-labeled aptamer with concentration of 1  $\mu$ M was injected for 10 min (10  $\mu$ l/min, 25 °C) and then followed by 5 min biotin (10  $\mu$ M) blocking.

For binding assay, HA proteins with different concentration (2, 5, 10, 20 and 40  $\mu\text{g/mL}$ ) were injected onto the sensor chip and the affinity binding was monitored for 2 min followed by washing with running buffer.

The binding reaction equation of two macromolecular interactants (A and B) for a one-to-one binding reaction is written as :



$$K_A = K_a / K_d = [AB]/[A][B]$$

$$K_D = K_d / K_a = [A][B]/[AB]$$

Where  $K_a$  is the association rate constant in  $\text{M}^{-1}\text{s}^{-1}$  and  $K_d$  is the dissociation rate constant in  $\text{s}^{-1}$ .  $K_A$  and  $K_D$  are the equilibrium constants,  $K_A$  is the association constant in  $\text{M}^{-1}$  and  $K_D$  is the dissociation constant in  $\text{M}$ .

After measuring SPR signals, the association and dissociation rate constants ( $K_a$  and  $K_d$ ), and equilibrium association constants ( $K_A$ ) and dissociation constant ( $K_D$ ) were calculated by a 1:1 [Langmuir] fitting model using the Qdata analysis software.

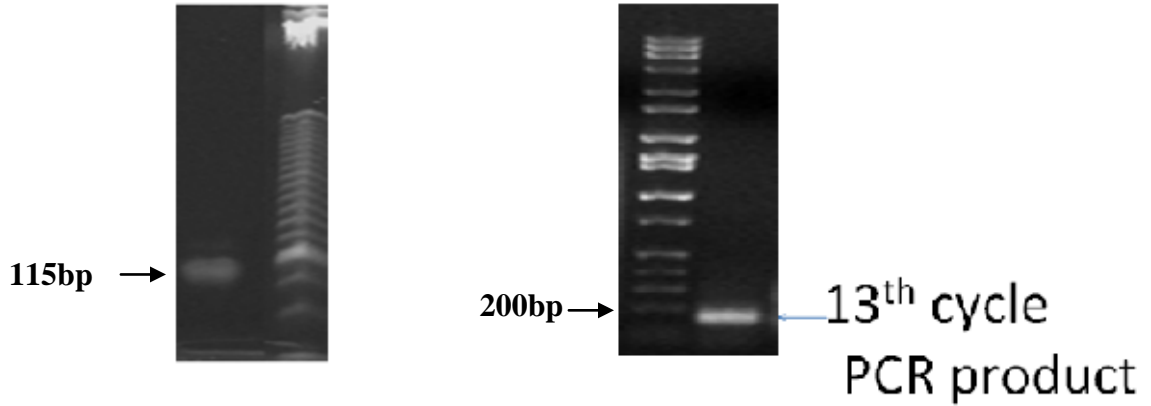
Further experiments were conducted to study the binding affinity between the selected aptamer and the H5N1 virus with different titer of 0.064, 0.128, 0.32 and 0.64 HAU. Then the specificity was investigated by testing non-target AI virus H5N2, H5N9, H9N2, H7N2 and H2N2 with titer of 0.64 HAU.

## **CHAPTER 5 RESULTS AND DISCUSSION**

## 5.1 Selection of DNA Aptamers

We performed an in vitro selection approach with a pool of DNA sequences totally randomized at 74 nt to select DNA aptamers that bind to AIV H5N1. Purified H5N1 strain (HA titer:128) and a DNA library of about  $10^{14}$  different molecules with a length of 115 nt randomized at central 74 nt (pool 74) were subjected to in vitro selection. The H5N1-binding DNA aptamers were selected by incubating HA with the DNA pool and subsequent nitrocellulose filtration. HA-bound aptamers were eluted from the filters, and then amplified by PCR.

Individual single-stranded aptamer DNAs were derived from an aliquot of this dsDNA stock by  $\lambda$  digest. Lambda exonuclease is a highly processive 5' to 3' exodeoxyribonuclease that selectively digests the 5'-phosphorylated strand of dsDNA. Therefore, a 5'-phosphorylated group was introduced into one strand of dsDNA by performing PCR where only reverse primer was 5'-phosphorylated. As a result, this phosphorylated strand was then removed by digestion with lambda exonuclease while the other biotinylated strand was retained. The single strand DNA was used as the input for the next selection cycle. All DNAs were quantified by spectrophotometer and analyzed by PAGE gel (Figs. 5.1 and 5.2).



M 1  
M: 25bp ladder  
1: 13<sup>th</sup> cycle ds-DNA aptamers

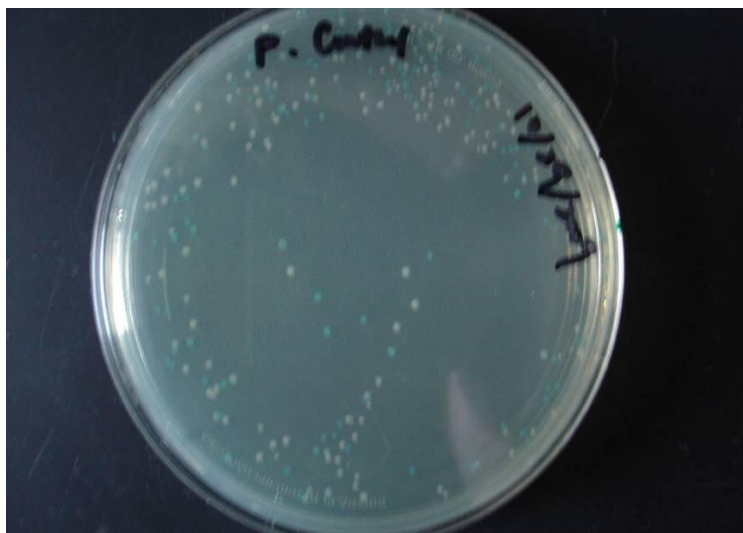
**Fig. 5.2** PAGE gel of 13<sup>th</sup> cycle ds-DNA aptamers selected by SELEX

1 M  
M: 25bp ladder;  
1: 3<sup>rd</sup> cycle ds-DNA aptamers

**Fig. 5.1** PAGE gel of 3<sup>rd</sup> cycle ds-DNA aptamers selected by SELEX

## 5.2 Cloning of DNA Aptamers

After 13 rounds of selection, ssDNA pool was purified using the Qiaquick PCR Purification Kit (Qiagen, Germany). The purified products were ligated to pGEM-T easy vectors (Promega), according to the manufacturer’s instructions. Plasmids from individual white clones were ready to be sequenced. Fig. 5.3 shows screening results with controls.



**Fig. 5.3 (a)**

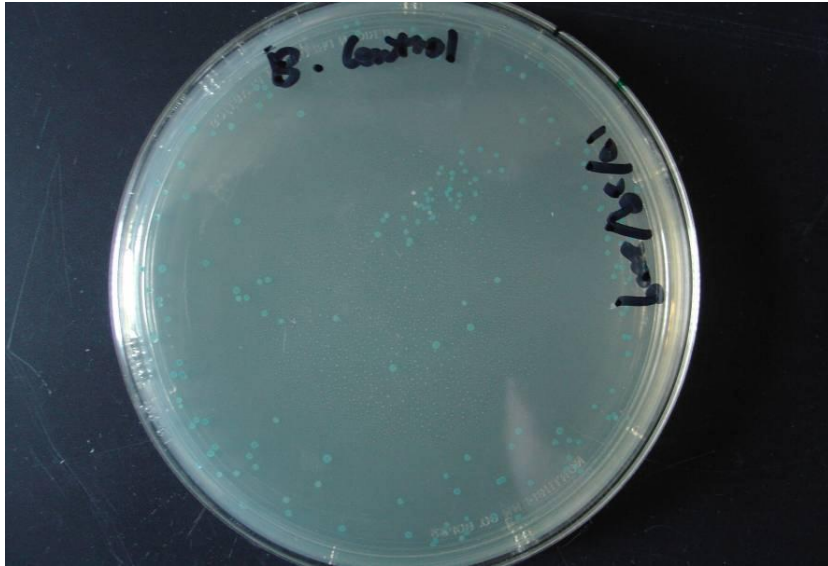


Fig. 5.3 (b)

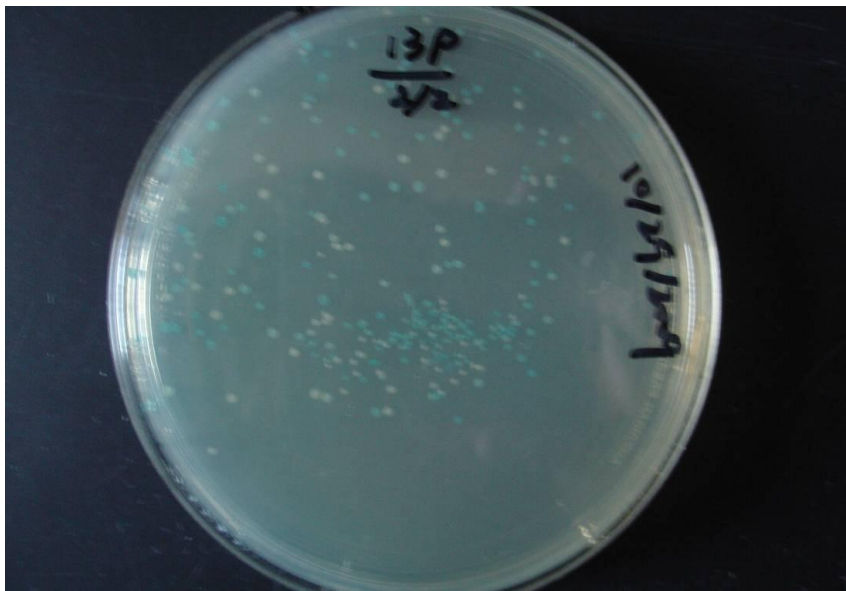
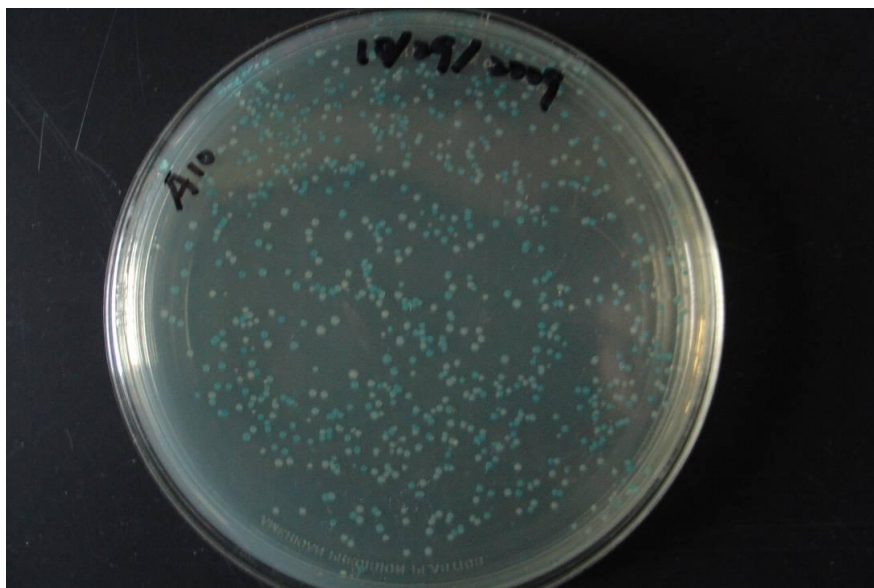


Fig. 5.3 (c)



**Fig. 5.3 (d)**

**Fig. 5.3** Blue/white screening test for 13<sup>th</sup> cycle aptamers recombinants on LB agar plate 1 (c) and plate 2 (d) with positive control (a) and background control (b).

pGEM®-T Easy Vectors are linearized vectors with a single 3' terminal thymidine at both ends. The T-overhangs at the insertion site enhanced the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. Since pGEM®-T Easy Vectors are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the  $\alpha$ -peptide coding region of the enzyme  $\beta$ -galactosidase, we chose pGEM®-T Easy Vectors transformation system to screen recombinants. Insertional inactivation of the  $\alpha$ -peptide allows identification of recombinants by blue/white screening on indicator plates.

As shown in Fig. 5.3 (a), positive control recombinants which contain a 542 bp fragment from pGEM®-luc Vector DNA appeared high ratio of white colonies. This vector DNA sequence has been mutated to contain multiple stop codons in all six reading frames, which



ensures a low background of blue colonies for the control reaction. On the other hand, Fig. 5.3 (b) indicates high efficiency competent cell JM109 works well on efficiency in the term of high background of blue colonies.

We picked up 30 white colonies randomly from each LB agar plate of blue/white screening test for 13<sup>th</sup> cycle aptamers recombinants (Figs. 5.3 (c) and 5.3 (d))colonies were grown in LB media containing 50 µg/mL ampicillin (see Appendix B), and plasmid DNA was extracted with a Miniprep Kit (Qiagen). After evaluation of PCR results, aptmaers insert in the plasmid DNA of each clone were sequenced.

### **5.3 Sequencing of DNA Aptamers**

Since there are some repeat sequences and wrong matches, we got three ssDNA aptamers sequences out of all positive colonies ultimately. Three sequences are shown below.

#### **O16**

**GTGTGCATGGATAGCACGTAACGGTGTAGTAGATACGTGCGGGTAGGAAGAAA  
GGGAAATAGTTGTCCTGTTG**

#### **O20**

**GGCCGAATTGGTTCGTCGAGCGAGTCACACCAACAATGCTGCGATAGAACTT  
CGTACGAGCTTTCTTACGCTG**

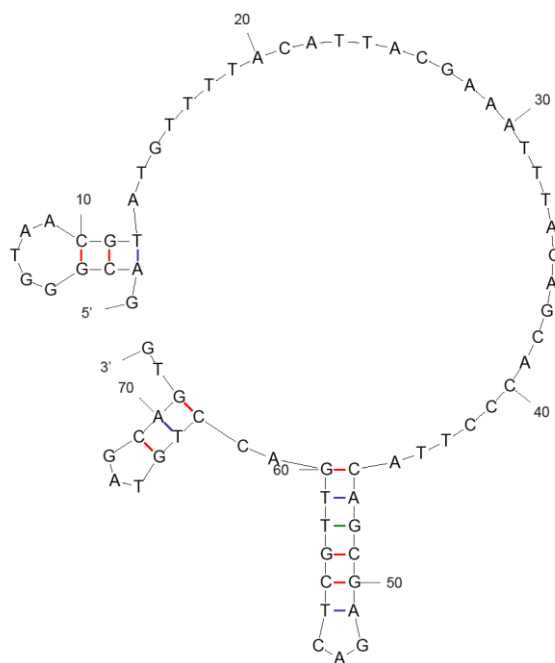
#### **O10**

**GACGGGTAACGTATGTTTTACATTACGAAATTTAGAGCACCCCTTACAGCGAGAC  
TCGTTGACCTGTAGCAGTG**

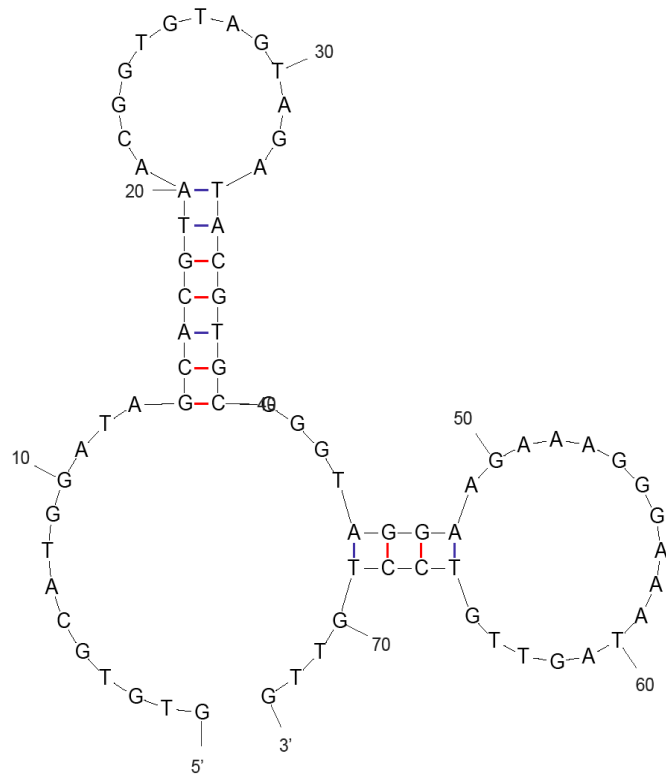
Considering the low rate of sequencing selection, we concentrated on these three sequences above and found that O20 has 74-nucleotide sequence while the other two have 73-nucleotide sequences.

O10 and O16's sequences show that it is unreasonable to obtain 73-nucleotide sequences of both. According to the mutagenic PCR theory (Cadwell et al., 2010), the error rate of Taq polymerase is high in the range  $0.1 \times 10^{-4}$  to  $2 \times 10^{-4}$  per nucleotide per pass of the polymerase. So we can assume that in the amplification process, target aptamers O10 and O16 have mutation which changes 74-nucleotide sequences to 73-nucleotide sequences.

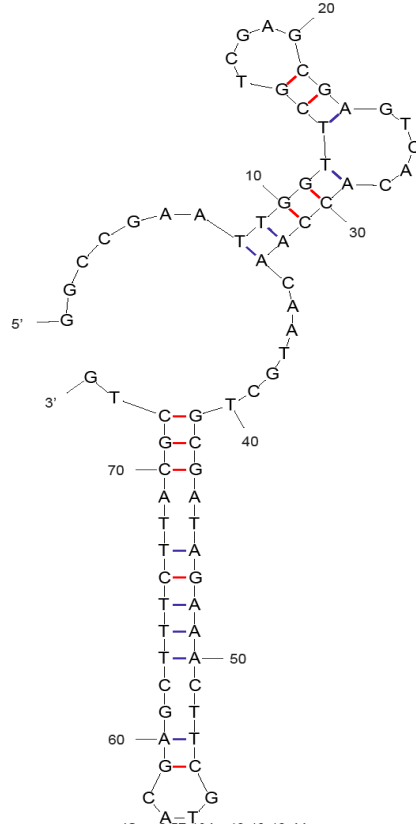
#### 5.4 Secondary Structure Prediction of DNA Aptamers



**Fig. 5.4 (a)**



**Fig. 5.4 (b)**



**Fig. 5.4 (c)**

**Fig. 5.4** Secondary structure of aptamer O13 (a), aptamer O16 (b) and aptamer O20 (c) predicted by Mfold tool (<http://mfold.bioinfo.rpi.edu/>)

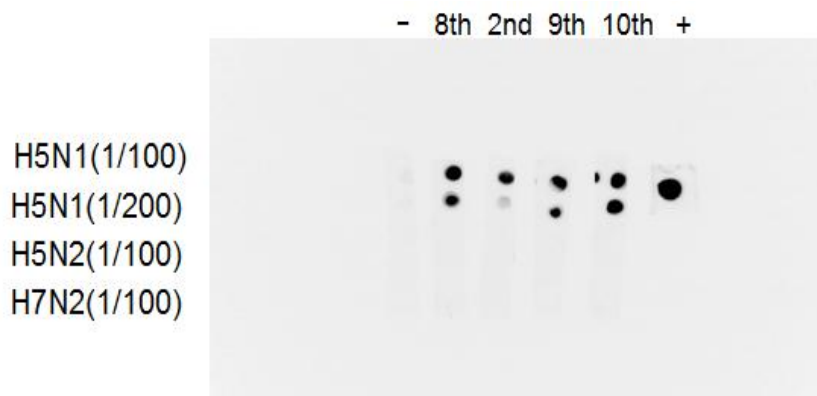
The aptamers sequences were analyzed and the secondary structures were predicted by means of the free energy minimization algorithm according to Zuker (2003) using the web server based mfold tool (<http://mfold.bioinfo.rpi.edu/>).

From Fig. 5.4 (a), a typical stem and loop motif was seen in the secondary structure of aptamers O13, which is predicted to be the binding region for the target. Aptamer O16 (Fig. 5.4 (b)) represented the most common stem-loop style which connect several loops with stems together. However, aptamer O20 shows long stem and small loop structure (Fig. 5.4 (c)).

In general, the secondary structures of the aptamers are folded into a long stem - loop structure with a large internal loop and a couple of small bulges or loops.

## 5.5 Affinity and Specificity Evaluation of DNA Aptamers

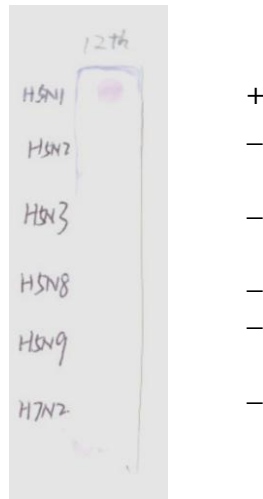
### 5.5.1 Dot Blot Test Results



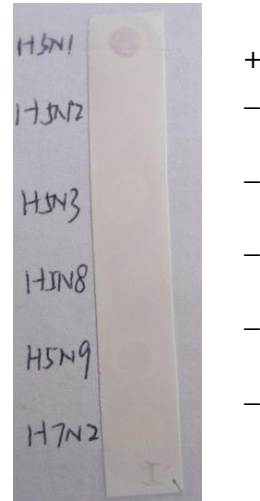
**Fig. 5.5** Dot Blot results of 2<sup>nd</sup>, 8<sup>th</sup>, 9<sup>th</sup>, and 10<sup>th</sup> cycle ss-DNA aptamers pool reacted with H5N1/H5N2/H7N2 virus

First of all, Dot Blot test was conducted to check several rounds of aptamers pools' specificity and affinity. In Fig. 5.5, 2<sup>nd</sup>, 8<sup>th</sup>, 9<sup>th</sup>, and 10<sup>th</sup> cycle ssDNA pools reacted with 1/100 and 1/200 diluted H5N1 (128 HA) as well as H5N2 and H7N2 virus. Each cycle of ssDNA pool has specificity to H5 compared to H7 subtypes, and they also show specificity to H5N1 than H5N2 because ssDNA pool has little reaction with H5N2.

### 5.5.2 Dot ELISA Test Results



**Fig. 5.6 (a)**



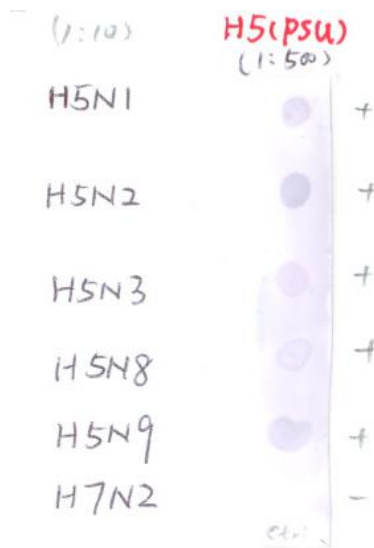
**Fig. 5.6 (b)**

**Fig. 5.6** Dot ELISA results demonstrate 12<sup>th</sup> cycle ssDNA (a) and 13<sup>th</sup> cycle ssDNA (b) pool's affinity with subtype H5N1, H5N2, H5N3, H5N8, H5N9, H7N2 virus.

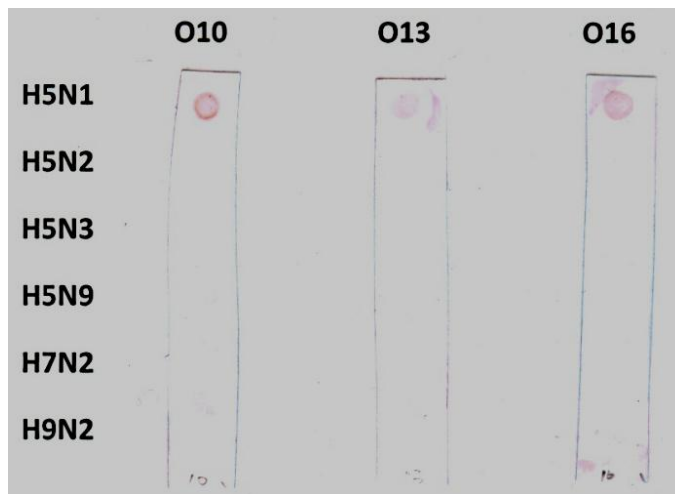
A newly developed Dot-ELISA, rather than standard Sandwich-ELISA, was used to detect aptamers pools' affinity. In the Dot-ELISA, viruses were immobilized on nitrocellulose strips which can detect aptamer targeted to virus. Then these attached biotin-labeled aptamer can react with streptavidin-AP conjugate through biotin- streptavidin interaction. The amount of enzyme linked conjugate bound is assayed by incubating the strip with an appropriate chromogenic substrate leading a color development. Therefore ssDNA pools were deemed to react with AI virus when strips appear purple dot on it. From comparison of 12<sup>th</sup> and 13<sup>th</sup> cycle ssDNA pool's affinity with subtype H5N1, H5N2, H5N3, H5N8, H5N9, H7N2 in Fig. 5.6, It's easy to conclude that ssDNA pools indicate strong specificity to H5N1 but little cross-interaction with other subtypes.

Upon quick and effective outcome of Dot ELISA test, specificity and affinity test for individual aptamers were continued to take.

### 5.5.2.1 Apatmer Specificity



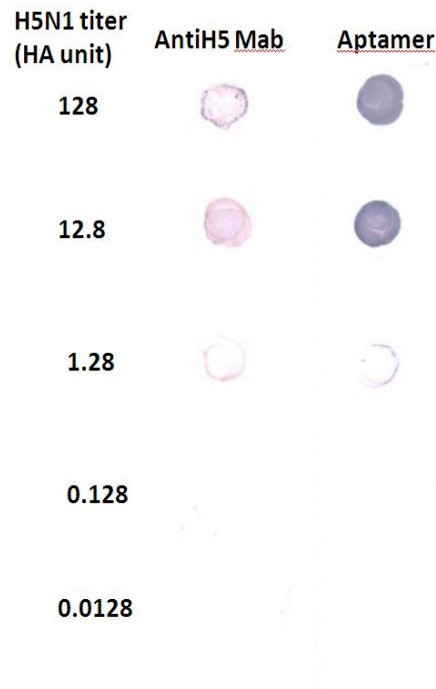
**Fig. 5.7** Specificity of monoclonal anti-H5 antibody to different H5 and H7 subtypes tested by Dot ELISA as control



**Fig. 5.8** Specificity of aptamers to different H5/H7/H9 subtypes tested by Dot ELISA

After purification of three ssDNA aptamers, Dot ELISA test is applied first for their specificity test. As a comparison control shown in Fig. 5.7, anti-H5 monoclonal antibody (4.4. µg/mL from ADL, Penn State University) shows specificity on H5 subtypes and has no interaction with H7 subtype. Besides, aptamers O10, O13, O16 not only show specificity to H5 subtype but also appear unique interaction with H5N1 (Fig. 5.8). Among those three aptamers, O16 performed best affinity on H5N1 according to their darkness of dot. From the above figures, we can make a preliminary conclusion that these three aptamers has good specificity to H5N1.

### 5.5.2.2 Aptamer Affinity



**Fig. 5.9** Aptamer O16's affinity to H5N1 compared with anti H5 monoclonal antibodies' affinity to H5N1

As the one of the best candidate aptamers, O16 was selected for further affinity test. In this Dot ELISA affinity test, H5N1 virus was diluted from 128 HA units to 0.0128 HA units. One g/ml O16 aptamer ssDNA was applied on the strip. The result shows the minimum virus concentration which can interact with aptamers O16 at that concentration is between 0.128 and 1.28 HA unit.

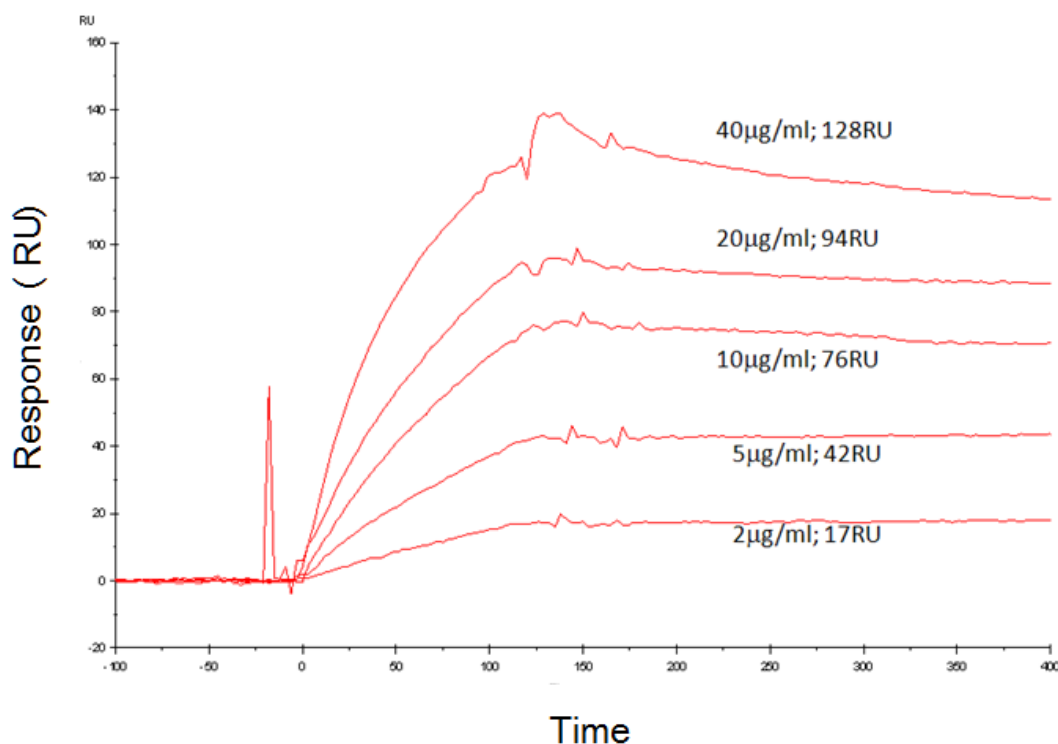
Meanwhile, anti-H5 monoclonal antibodies at the same concentration to aptamers present almost the same minimum virus concentration of ELISA interaction. But they showed greatly less interaction with H5N1 than aptamers due to color comparison in Fig.5.9. Therefore, we can conclude that aptamer O16 represent equal even better affinity to H5N1 with contrast to anti-H5 monoclonal antibody.



Considering that the result cannot be quantitative and not be compared with antibody's affinity, further binding test and characterization of aptamers will be continued.

### 5.5.3 SPR Test Results\*

The chip was ready for binding measurements after washing with running buffer. Aptamer was immobilized on Channel 1 and channel 2 without immobilization was used as a reference channel. The immobilization of aptamer resulted in increased 997 RU SPR signal.



**Fig. 5.10** An overlay of the SPR signals from the interaction between HA protein and aptamer

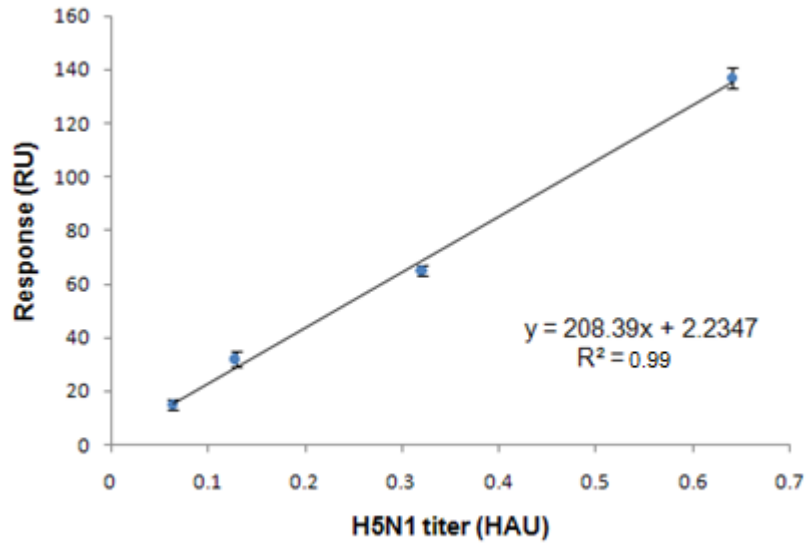
After HA proteins with different concentration (2, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) were injected onto the sensor chip, the analytical signal, recorded in resonance units (RU), was computed as

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\* Results presented are from SPR test that was conducted by Dr. Ronghui Wang. Due to my healthy reason, I was absent from this experiment.

the difference between the aptamer and corresponding reference channel. We evaluated the affinity binding of HA protein to the selected aptamer by injecting increasing concentrations of HA over the aptamer and reference surfaces. In this way, the portion of the SPR signal that was attributed to specific affinity binding could be calculated by subtracting the non-specific binding and bulk refractive index effects detected on the reference surface from the total SPR signal measured on the aptamer surface. An overlay of the sensor SPR signals from the interactions between HA and the selected aptamer is shown in Fig. 5.10. As expected, injection of increasing HA concentrations resulted in an increase of the SPR signal. Analysis of data for interaction between HA and aptamer; association and dissociation rate constants ( $K_a$  and  $K_d$ ), and equilibrium association constants ( $K_A$ ) were calculated by a 1:1 [Langmuir] fitting model using the Qdata analysis software. The calculated  $K_a$  and  $K_d$  was  $4.69 \times 10^4$  ( $M^{-1} s^{-1}$ ) and  $2.18 \times 10^{-4}$  ( $s^{-1}$ ), respectively. So  $K_A$  was  $2.15 \times 10^8$  ( $M^{-1}$ ) and  $K_D$  was  $4.65 \times 10^{-9}$  (M), indicating strong binding between the HA protein and the selected aptamer.

The inactivated AI H5N1 virus with different titer of 0.064, 0.128, 0.32 and 0.64 HAU in buffer solution was tested using SPR. The result was shown in Fig. 5.11. The Standard Deviations (SDs) was indicated as error bars in the figure. A linear relationship was found and the corresponding equation was described as:  $y=208.39x +2.2347$  ( $R^2=0.99$ ), where y (SPR signal) was expressed in RU and x (virus titer) in HAU. The result showed that the selected aptamer could be used as a ligand to detect target AIV H5N1.



**Fig. 5.11** Detection of inactivated AIV H5N1 using the aptamer immobilized SPR

In specificity test non-target AI virus H5N2, H5N9, H9N2, H7N2 and H2N2 with titer of 0.64 HAU were investigated. No detectable signal was obtained with AIV H5N9, H9N2, H7N2 and H2N2. However, 33 RU SPR signal was observed when detection of AIV H5N2, showing some cross-interaction with subtype H5N2.

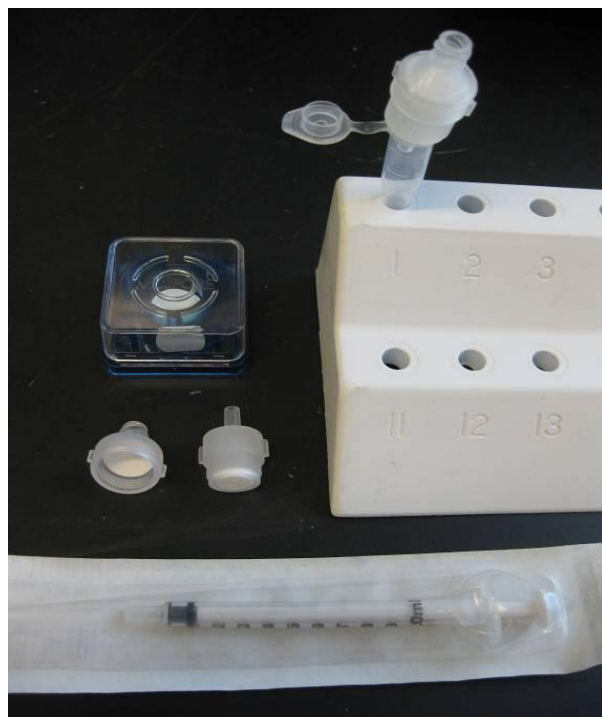
## **CHAPTER 6 CONCLUSIONS AND FUTURE RESEARCH**

In this study, Systematic Evolution of Ligands by EXponential enrichment (SELEX) was used to select DNA aptamers targeted to hemagglutinin (HA) and neuraminidase (NA) proteins of AI H5N1 virus. After 13 cycles of isolation, 115 bp DNA-aptamers were screened out and three aptamer sequences were obtained after cloning. The results of Dot ELISA, Dot Blot tests showed that those DNA-aptamers have stronger binding specificity to AI H5N1 subtype compared with their binding to H5N2, H5N3, H5N9, H2N2, H7N2 and H9N2. Aptamers also presented stronger binding affinity to AIV H5N1 than monoclonal anti-H5 subtype antibodies.

In further evaluation process, saturation curve was obtained in SPR analysis by plotting the response units (RU) as a function of H5N1 or HA protein concentration. The calculated  $K_a$  and  $K_d$  was  $4.69 \times 10^4$  ( $M^{-1} s^{-1}$ ) and  $2.18 \times 10^{-4}$  ( $s^{-1}$ ), respectively. The  $K_A$  was  $2.85 \times 10^8$  ( $M^{-1}$ ) and  $K_D$  was  $4.65 \times 10^{-9}$  (M), indicating strong binding between the HA protein and the selected aptamer. A linear equation between y (SPR signal) in RU and x (virus titer) in HAU was described as:  $y=208.39x + 2.2347$  ( $R^2=0.99$ ), demonstrating that the selected aptamers O16 could be used as a ligand to detect target AIV H5N1. Specificity test by SPR showed weak cross-interaction with AI subtype H5N2.

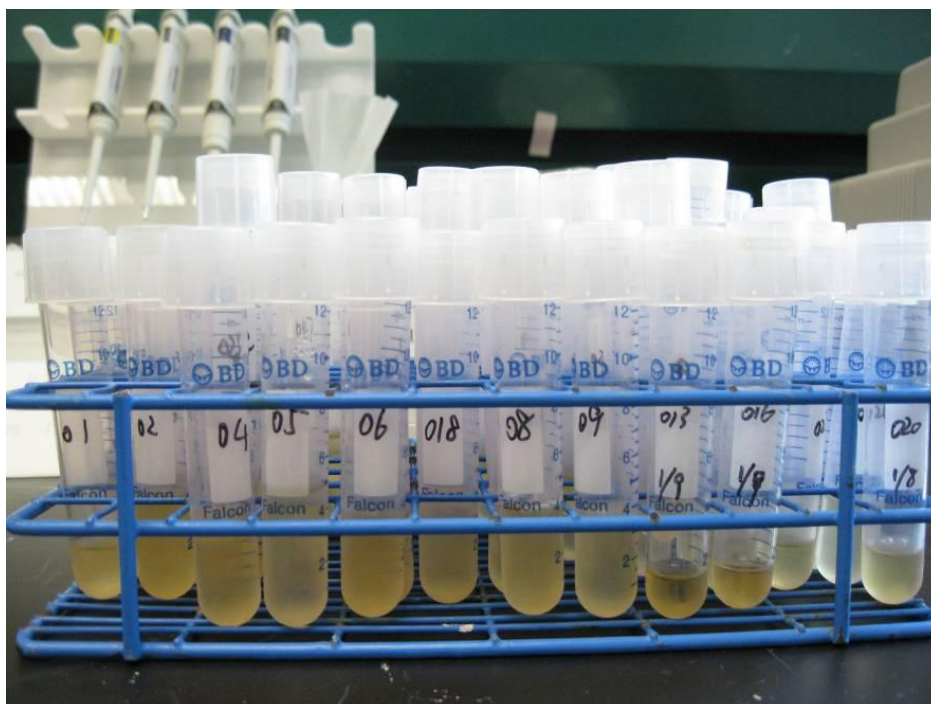
In the future research, aptamers selected in this study could be used in assays as a specific recognition element for sensitive, specific and rapid detection of AI H5N1 virus.

## APPENDIX A. Nitrocellulose Filter Binding Test in SELEX



**Fig.A.1** Nitrocellulose Filter binding test equipment

## APPENDIX B. Selected Aptamers Cloning Test



**Fig.B.1** Selected aptamers colonies grown in LB medium

## REFERENCES

- Alexander, D.J. 2000. A review of avian influenza in different bird species. *Vet Microbiol.*74:3-13
- Alocilja, E.C., Radke, S. 2003. Market analysis of biosensors for food safety. *Biosens. Bioelectron.* 18(5-6):841-846.
- Baskerville, S., Zapp, M., Ellington, A.D. 1999. Anti-Rex aptamers as mimics of the Rex-binding element. *J. Virol.* 73:4962–4971.
- Bini, A., Minunni, M., Tombelli, S., Centi, S., Mascini, M. 2007. Analytical performances of aptamerbased sensing for thrombin detection. *Anal. Chem.* 79: 3016-3019.
- Boltovets, P.M., Boyko, V.R., Kostikov, I.Y., Dyachenko, N.S., Snopok, B.A., Shirshov, Y.M. 2002. Simple method for plant virus detection: effect of antibody immobilization technique. *J Virol Methods* 105:141–146.
- Boon, A.C., French, A.M., Fleming, D.M., Zambon, M.C. 2001. Detection of influenza a subtypes in community-based surveillance. *J. Med.Virol.* 65:163-170.
- Brody, E.N., Gold, L. 2000. Aptamers as therapeutic and diagnostic agents. *Rev. Mol. Biotechnol.* 74 :5–13.
- Cadwell, R.C., Joyce, G.F., 1994. Mutagenic PCR. *Genome Res.* 3:S136-S140.
- Capua, I., Alexander, D.J. 2004. Avian influenza: Recent developments. *Avian Pathol.* 33(4):393-404.
- Capua, I., Marangon, S. 2006. Control of avian influenza in poultry. *Emerg.Infect. Dis.* 12:1319-1324.
- CDC 2007 <http://www.cdc.gov/flu/professionals/diagnosis/rapidclin.htm>
- Chan, W.C., Maxwell, D.J., Gao, X., Bailey, R.E., Han, M., Nie, S. 2002. Luminescent quantum dots for multiplexed biological detection and imaging. *Curr. Opin. Biotechnol.* 13: 40-46.
- Chen, J., Jiang, J., Gao, X., Liu, G., Shen, G., Yu, R. 2008. A new aptameric biosensor for cocainebased on surface enhanced Raman scattering spectroscopy. *Chemistry* 14: 8374-8382.
- Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., Ingber, D.E. 1998. Micropatterned surfaces for control of cell shape, position and function. *Biotechnol. Prog.*14: 356.
- Cheng, C., Dong, J., Yao, L., Chen, A., Jia, R., Huan, L., Guo, J., Shu, Y., Zhang, Z. 2008. Potent inhibition of human influenza H5N1 virus by oligonucleotides derived by SELEX. *Biochemical and Biophysical Research Communications* 366 : 670 – 674.



- Choi, J.H., Chen, K.H., Strano, M.S. 2006. Aptamer-capped nanocrystal quantum dots: a new method for label-free protein detection. *J. Am. Chem. Soc.* 128: 15584-15585.
- Clark, S.L., Remcho, V.T. 2002. Aptamers as analytical reagents. *Electrophoresis* 23: 1335–1340.
- Collett, J. R., Cho, E. J., Ellington, A. D. 2005. Production and processing of aptamer microarrays. *Methods* 37: 4–15.
- Cooper, M.A. 2003. Label-free screening of bio-molecular interactions. *Anal Bioanal Chem* 377:834–842
- Cox, J.C., Ellington, A.D. 2001. Automated selection of anti-proteins aptamers. *Bioorg. Med. Chem.* 9: 2525–2531.
- Cox, J.C., Hayhurst, A., Hesselberth, J., Davidson, E.A., Sooter, L.J., Bayer, T.S., Georgiou, G., Ellington, A.D., 2002a. Automated selection of aptamers against protein targets translated in vitro: from gene to aptamer. *Nucl. Acids Res.* 30:108.
- Cox, J.C., Rajendran, M., Riedel, T., Davidson, E.A., Sooter, L.J., Bayer, T.S., Schmitz-Brown, M., Ellington, A.D., 2002b. Automated acquisition of aptamer sequences. *Comb. Chem. High. T. Scr.* 5:289–299
- Cram, P., Blitz, S.G., Monto, A., Fendrick, A.M. 1999. Diagnostic testing for influenza: review of current status and implications of newer treatment options. *The American Journal Of Managed Care* 5: 1555-1561.
- Critchley, P., Dimmock, N.J. 2004. Binding of an influenza A virus to a neomembrane measured by surface plasmon resonance. *Bioorg Med Chem* 12:2773–2780.
- Dunn, P.A., Wallner-Pendleton, E.A., Lu, H., Shaw, D.P., Kradel, D., Henzler, D.J., Miller, P., Key, D.W., Ruano, M., Davison, S. 2003. Summary of the 2001-02 Pennsylvania H7N2 low pathogenicity avian influenza outbreak in meat type chickens. *Avian Dis.* 47 (3suppl):812-816.
- Elghanian, R., Storhoff, J.J., Mucic, R.C., Letsinger, R.L., Mirkin, C.A. 1997. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science* 277: 1078-1081.
- Ellington, A.D., Szostak, J.W., 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature* 346: 818–822.
- Ellington, A.D., Szostak, J.W., 1992. Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature* 355: 850–852.
- Famulok, M., Mayer, G., Blind, M., 2000. Nucleic acid aptamers – from selection in vitro to application in vivo. *Acc. Chem. Res.* 33: 591–599.

- Famulok, M., Szostak, J.W., 1992. Stereospecific recognition of tryptophan agarose by in vitro selected RNA. *J. Am. Chem. Soc.* 114: 3990–3991.
- Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype(H16) obtained from black-headed gulls. *J. Virol.* 79 (5):2814-22.
- Glick G.D., Toogood, P.L., Wiley, D.C., Skehel, J.J, Knowles, J.R. 1991. Ligand recognition by influenza virus. The binding of bivalent sialosides. *J Biol Chem.* 266 (35):23660-9.
- Golden, M.C., Collins, B.D., Willis, M.C., Koch, T.H., 2000. Diagnostic potential of PhotoSELEX-evolved ssDNA aptamers. *J. Biotechnol.* 81:167–178.
- Gronewold, T.M., Glass, S., Quandt, E., Famulok, M. 2005. Monitoring complex formation in the blood-coagulation cascade using aptamer-coated SAW sensors. *Biosens. Bioelectron.* 20: 2044-2052.
- Harada K., Frankel A.D., Jenison, R.D., Gill, S.C., Pardi, A., Polisky, B. 1994. High-resolution molecular discrimination by RNA. *Science* 263: 1425–1429.
- Heidenreich, O., Eckstein, F., 1992. Hammerhead ribozyme-mediated cleavage of the long terminal repeat RNA of human immunodeficiency virus type 1. *J. Biol. Chem.* 267: 1904–1909.
- Herman, T., Patel, D. 2000. Adaptive recognition by nucleic acid aptamers. *Science* 287: 820–825.
- Homola, J. 2003. Present and future of surface plasmon resonance biosensors. *Anal Bioanal Chem* 377:528–539
- Horimoto, T., Kawaoka, Y. 2005. Influenza: lessons from past pandemics, warning from current incidents. *Nat. Rev. Microbiol.* 3:591-600.
- Jayasena, S. 1999. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin. Chem.* 45: 1628–1650.
- Jenison, S., Yamada, T., Morris, C., Anderson, B., Torrezmartinez, N., Keller, N. & Hjelle, B. 1994. Characterization of human antibody responses to Four Corners hantavirus infections among patients with hantavirus pulmonary syndrome. *J. Virology* 68: 3000-3006.
- Klug, S.J., Huttenhofer, A., Famulok, M. 1999. In vitro selection of RNA aptamers that bind special elongation factor SelB, a protein with multiple RNA-binding sites, reveals one major interaction domain at the carboxyl terminus. *RNA* 5: 1180–1190.
- Kortt, A.A., Nice, E., Gruen, L.C. 1999. Analysis of the binding of the Fab fragment of monoclonal antibody NC10 to influenza virus N9 neuraminidase from tern and whale using the

BIAcore biosensor: effect of immobilization level and flow rate on kinetic analysis. *Anal Biochem* 273:133–141

Lazcka, O, Del Campo, F.J., Muñoz, F.X. 2007. Pathogen detection: a perspective of traditional methods and biosensors. *Biosens. & Bioelectron.* 22(7):1205-17.

Lee, S.J., Youn, B.S., Park, J.W., Niazi, J.H., Kim, Y.S., Gu, N.B. 2008. ssDNA Aptamer-based surface Plasmon Resonance Biosensor for the Detection of Retinol binding Protein 4 for the early Diagnosis of Type 2 Diabetes. *Anal. Chem.* 80:2867-2873.

Levy, M., Cater, S.F., Ellington, A.D. 2005 Quantum-dot aptamer beacons for the detection of proteins. *Chembiochem* 6: 2163-2166.

Liu, J., Lu, Y. 2006 Fast Colorimetric Sensing of Adenosine and Cocaine Based on a General Sensor Design Involving Aptamers and Nanoparticles. *Angew. Chem.* 118: 96-100.

Lu H. 2003. A longitudinal study of a novel Dot- Enzyme-linked immunosorbent assay for detection of avian influenza virus. *Avian Disease* 47:361-369.

Lu, Y., Yang, Y., Sellinger, A., Lu, M., Huang, J., Fan, H., Haddad, R., Lopez, G., Burns, A.R., Sasaki, D.Y., Shelnett, J., Brinker, C.J. 2001. Self-Assembly of Mesoscopically Ordered Chromatic Polydiacetylene/Silica Nanocomposites. *Nature* 410:913–917.

Luzi, E., Minunni, M., Tombelli, S., Mascini, M., 2003. New trends in affinity sensing: aptamers for ligand binding. *Trends Anal. Chem.* 22: 810–818.

Marshall, K.A. and Ellington, A.D. 2000. In vitro selection of RNA aptamers. *Methods Enzymol.* 318:193-214.

McKimm-Breschkin, J.L., Colman, P.M., Jin, B., Krippner, G.Y., McDonald, M., Reece, P.A., Tucker, S.P., Waddington, L., Watson, K.G., Wu, W.Y. 2003. Tethered neuraminidase inhibitors that bind an influenza virus: a first step towards a diagnostic method for influenza. *Angew Chem. Int. Ed. Engl.* 42 : 3118–3121.

Mok, W., Li, Y.F. 2008. Recent progress in nucleic acid aptamer-based biosensors and bioassays. *Sensors* 8 : 7050–7084.

Mounts, A.W., Kwong, H., Izurieta, H.S., Ho, Y., Au, T., Lee, M. 1999. Case-control study of risk factors for avian influenza A(H5N1) disease, Hongkong. *J. Infect. Dis.* 180:505-508

Murphy, B.R., Webster, R.G. 1996. Orthomyxoviruses, p. 1397-1445. In *Fields Virology*, 3<sup>rd</sup> ed., B.N. Fields, D.M. Knipe, and P.M. Howley (ed.) Lippincott-Raven, Philadelphia, PA.

Mullett, W.M., Lai, E.P., Yeung J.M. 2000. Surface plasmon resonance-based immunoassays. *Methods* 22:77–91

- Nice, E.C., McInerney, T.L., Jackson D.C. 1996. Analysis of the interaction between a synthetic peptide of influenza virus hemagglutinin and monoclonal antibody using an optical biosensor. *Mol Immunol* 33:659–670
- Nutiu, R., Li, Y. 2005 Aptamers with fluorescence-signaling properties. *Methods* 37: 16-25
- Okamoto T., Yamaguchi I. 2003. Optical absorption study of the surface plasmon resonance in gold nanoparticles immobilized onto a gold substrate by self-assembly technique. *J. Phys. Chem.* 107: 10321-10324.
- Ostuni, E., Whitesides, G.M. 1999. The interaction of proteins and cells with self-assembled monolayers of alkanethiolates on gold and silver. *Colloids and Surfaces B: Biointerfaces* 15:3.
- Owen, C.S. 1986. Magnetic sorting of Leukocytes. *Cell Biophy.* 8:287-296
- Pappas, M.G., Hajkowskia, R., Hockmeyer, W.T. 1983. Dot enzyme-linked immunosorbent assay (Dot-ELISA): a micro technique for the rapid diagnosis of visceral leishmaniasis. *J.of Immuno. Methods.* 64:205-214.
- Patel, D.J., Suri, A.K., 2000. Structure, recognition and discrimination in RNA aptamer complexes with cofactors, amino acids, drugs and aminoglycoside antibiotics. *Rev. Mol. Biotechnol.* 74: 39–60.
- Paulson, J.C. 1985. The receptors. *Conn M.(ed.). Academic Press* 2:131-219.
- Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. 1991. Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. *Science* 253 : 314–317.
- Rao, C.N.R., Kulkarni, G.U., Thomas, P.J., Edwards, P.P. 2000. Metal nanoparticles and their assemblies. *Chem. Soc. Rev.* 29: 27-35.
- Rogers, G.N., Paulson, J.C., 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology.* 127 (2):361-73.
- Rogers, G.N., Paulson, J.C., Daniels, R.S., Skehel, J.J., Wilson, I.A. Wiley, D.C. 1983. Single amino acid substitutions in influenza hemagglutinin change receptor binding specificity. *Nature* 304(5921):76-8.
- Sambles J.R., Bradbery G.W., Yang F. 1991. Optical-excitation of surface-plasmons—an introduction. *Contemp. Phys.* 32:173–183.
- Sato, T., Ishii, M., Ohtake, F., Nagata, K., Terabayashi, T., Kawanishi, Y., Okahata, Y. 1999. Binding affinity of GM3 lactone for influenza virus. *Glycoconj J* 16:223–227.

- Saw, T.A., Lim, W., Shortridge, K., Tam, J., Liu, K.K., Mak, K.H. 1997, isolation of avian influenza A(H5N1) viruses from humans-Hongkong, May-December 1997. *MMWR Morb. Mortal. Wkly. Rep.* 46:1204-7.
- Schlenz, M.D., Gronewold, T.M.A., Tewes, M., Famulok, M., Quandt, E. 2004. A Love-wave biosensor using nucleic acids as ligands. *Sens. Actuator B* 101: 308-315.
- Schofield, D.J., Dimmock, N.J. 1996. Determination of affinities of a panel of IgGs and Fabs for whole enveloped (influenza A) virions using surface plasmon resonance. *J Virol Methods* 62: 33-42.
- Schurer, H., Stempera, K., Knoll, D., Mayer, G., Blind, M., Foerster, H., Famulok, M., Welzel, P., Hahn, U., 2001. Aptamers that bind to the antibiotic moenomycin A. *Bioorg. Med. Chem.* 92: 2557-2563.
- Song, J., Cheng, Q., Zhu S., Stevens, R.C. 2002. *Biomed Microdev* 4:213-221.
- Stamboulian, D., Bonvehi, P.E., Nacinovich, F.M., Cox, N. 2000. Influenza. *Infect. Dis. Clin. North Am.* 14:141-166.
- Su, C.C., Wu, T.Z., Chen, L.K., Yang, H.H., Tai, D.F. 2003. *Anal Chim Acta* 479:117-123.
- Takemoto, D.K., Skehel, J.J., Wiley, D.C. 1996. A surface plasmon resonance assay for the binding of influenza virus hemagglutinin to its sialic acid receptor. *Virology* 217:452-458.
- Tang, Q., Su, X., Loh, K.P. 2007. Surface plasmon resonance spectroscopy study of interfacial binding of thrombin to antithrombin DNA aptamers. *J. Colloid Interface Sci.* 315: 99-106.
- Tereshko, V., Skripkin, E., Patel, D.J., 2003. Encapsulating streptomycin within a small 40-mer RNA. *Chem. Biol.* 10: 175-187.
- Tombelli, S., Minunni, M., Luzi, E., Mascini, M. 2005. Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry* 67 : 135-141.
- Tuerk, C., Gold, L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249 : 505-510.
- Wang, L., Liu, X., Hu, X., Song, S., Fan, C. 2006 Unmodified gold nanoparticles as a colorimetric probe for potassium DNA aptamers. *Chem. Commun. (Camb.)*: 3780-3782.
- Wang, W., Chen, C., Qian, M., Zhao, X.S. 2008. Aptamer biosensor for protein detection using gold nanoparticles. *Anal. Biochem.* 373: 213-219.
- White, K., Sullenger, R.R., Rusconi, B.A., 2000. Developing aptamers into therapeutics. *J. Clin. Invest.* 106: 929-934.

Wilson, D.S., Szostak, J.W. 1999. In vitro selection of functional nucleic acids. *Annu Rev Biochem* 68: 611–647.

WHO 2007 [http://www.who.int/topics/avian\\_influenza/en/](http://www.who.int/topics/avian_influenza/en/)

Xiao, Y., Lubin, A.A., Heeger, A.J., Plaxco, K.W. 2005. Label-free electronic detection of thrombin in blood serum by using an aptamer-based sensor. *Angew. Chem. Int. Ed. Engl.* 44: 5456-5459.

You, K.M., Lee, S.H., Im, A., Lee, S.B., 2003. Aptamers as functional nucleic acids: in vitro selection and biotechnological applications. *Biotechnol. Bioprocess. Eng.* 8: 64–75.

Zhang, W.D. Evans, D.H. 1991. Detection and identification of human influenza viruses by the polymerase chain reaction. *J. Virol. Methods.* 33:165-189.

Zhao, S., Jin, M., Li, H., Tan, Y., Wang, G., Zhang, R., Chen, H. 2005. Detection of antibodies to the non-structural protein (NS1) of avian influenza viruses allows distinction between vaccinated and infected chickens. *Avian Dis.* 49:488-493.

Zhao, W., Chiuman, W., Brook, M.A., Li, Y. 2007. Simple and rapid colorimetric biosensors based on DNA aptamer and noncrosslinking gold nanoparticle aggregation. *ChemBiochem* 8: 727-731.

Zimmerman, J.M., Maher, L.J., 2002. In vivo selection of spectinomycinbinding RNAs. *Nucl. Acids Res.* 30: 5425–5435.

