ENHANCED DETECTION OF INFLUENZA WITH NANOTRAP PARTICLES

by

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## DEDICATION

This is dedicated to my always supportive and loving parents.

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| | |
|---|---|
| ATCC | American Type Culture Collection |
| DMEM | Dulbecco's Modified Eagle Medium |
| ELISA | enzyme-linked immunoassay |
| FBS | fetal bovine serum |
| HIV | Human Immunodeficiency Virus |
| HA | hemagglutinin |
| LFA | lateral flow assay |
| NA | neuraminidase |
| NP | nucleoprotein |
| NT | Nanotrap particles |
| PBS | phosphate buffered saline |
| qRT-PCR | quantitative real time PCR |
| RIDT | rapid influenza diagnostic test |
| RSV | Respiratory syncytial virus |
| RVFV | Rift Valley Fever Virus |
| VEEV | Venezuelan Equine Encephalitis Virus |

## ABSTRACT

ENHANCED DETECTION OF INFLUENZA WITH NANOTRAP PARTICLES

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George Mason University, 2015

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The Influenza virus is a leading cause of respiratory disease in the United States each year. The virus normally causes mild to moderate disease, however, hospitalization and death can occur in many cases. While there are several methodologies that are used for detection, problems such as decreased sensitivity and high false-negatives may arise. There is a crucial need for a fast, yet highly specific detection method. Nanotrap particles work to enrich whole virus and can be coupled to various downstream assays. Here, we demonstrate how Nanotrap particles with acrylic acid baits can be used to concentrate virus from high sample volumes and enhance detection up to 6-fold when coupled to plaque assays and qRT-PCR methodologies. The acrylic acid Nanotrap particles can concentrate virus from nasal fluid swab specimens and nasal aspirates. Importantly, the Nanotrap particles stabilize and protect the virus from degradation over extended periods of time and elevated temperatures. Lastly, in a coinfection scenario, other pathogens such as Coronavirus and *Streptococcus pneumoniae* do not interfere with

capture of Influenza virus. These results collectively demonstrate that Nanotrap particles are an important tool that can easily be integrated into various detection methodologies.

# CHAPTER ONE: STATEMENT OF PROBLEM

Diagnosis of early stage infections with viral pathogens is generally very difficult because patients typically present with nonspecific flu like symptoms. By the time a definitive diagnosis can be made using conventional methods, the infection may have already advanced to an untreatable, severe, or lethal stage. Thus, there is a substantial need for rapid, accurate, reliable, and safe diagnostic assays to guide physicians to administer treatment options (when available) as well as to notify public health officials if outbreaks are detected. As many pathogens can be transmitted through aerosol routes, the progression to disease will likely be accelerated underscoring the need to have diagnostic assays for early stage disease.

## CHAPTER TWO: SPECIFIC AIMS

The recent appearance and worldwide spread of the novel Influenza A (H1N1) virus has highlighted the need to reexamine the widely used Influenza diagnostic tests for their ability to detect viral antigens (e.g. nucleoprotein, hemagglutinin, and neuraminidase) in clinical specimens. Studies conducted by the CDC have demonstrated that while currently available diagnostic tests were capable of detecting Influenza A virus from respiratory specimens containing high levels of virus, sensitivity levels were significantly low (40%-69%) and decreased significantly as virus levels decreased[1]. Nanotrap particles are a novel technology that can address these critical sensitivity challenges. The Nanotrap particles contain internal affinity baits and work to sequester the target analyte and protect it from degradation. Targeted classes of analytes sequestered by the particles can be concentrated in small volumes to amplify the sensitivity of various assays[2–4] . We will utilize the Nanotrap particles to capture and detect Influenza using plaque assays to determine the capture of whole virus, quantitative real time PCR (qRT-PCR) to determine the capture of viral RNA, and western blotting to determine the capture of viral nucleoprotein (NP). The ability of the Nanotrap particles to capture ***whole virus*** allows Influenza A samples to be analyzed through numerous downstream analytical techniques.

Therefore, this proposal is based on the <u>rationale</u> that Nanotrap particles bind and concentrate the virus. Our <u>short-term goals</u> are to optimize capture, enrichment, and elution efficiencies of modified Nanotrap particles that are tailored for Influenza capture.

These Nanotrap particles will be able to not only enrich the virus, but also protect it from degradation over time. Our central hypothesis relevant to this proposal is that Nanotrap particles containing specific bait chemistries will be able to capture and enrich Influenza A and B viruses through the interaction of the viral surface proteins (hemagglutinin and neuraminidase) with the Nanotrap particle's shell and bait. This interaction will also protect the virus from degradation over time and at increased temperatures. Our short-term goals are to couple this novel diagnostic method to commercially available diagnostic methods to enhance viral detection at low concentration, and utilize the Nanotrap particles to protect the virus from degradation during transport. Our long-term goal is to develop a prototype specimen collection device for the enrichment and preservation of whole virus and viral analytes in clinical specimens.

**Aim 1: Optimize Nanotrap particles for the capture and enrichment of Influenza A and couple this method to commercially available, FDA-approved diagnostic assays.** **a)** We will determine which of the modified Nanotrap particles are optimal for Influenza capture at various viral titers. **b)** We will test the detection of Influenza A H1N1 (A/California/7/09) and Influenza B (B/Taiwan/2/62) in clinically relevant matrices and demonstrate that Nanotrap particles can increase sensitivity of qRT-PCR-based assays. **c)** We will determine the sensitivity of Nanotrap particle for Influenza capture in a mixed infection scenario by using our optimized Nanotrap particle to detect Influenza A in artificial nasopharyngeal samples spiked concurrently with Influenza and other viruses

found in nasopharyngeal samples. We will confirm these findings in three other Influenza A strains: A/Brisbane/10/2007, A/Brisbane/59/2007, and A/Texas/36/91.

**Aim 2: Demonstrate an increase in viral stability with the use of Nanotrap particles.** **a)** We will demonstrate that Nanotrap particles will protect the virus from degradation for up to 96 hours at an ambient temperature after specimen collection without the use of a cold-chain method. **b)** We will demonstrate the protection of the virus from degradation caused from high temperatures variation by exposing the Nanotrap-captured sample to an elevated temperature after specimen collection.

**Aim 3: Optimize Nanotrap particles for the capture of Influenza NP. a)** We will determine which of the Nanotrap particles best enhances the detection of Influenza A/Brisbane/10 NP. **b)** We will demonstrate that Nanotrap particles increase detection sensitivity of Influenza NP in clinically relevant matrices (nasal fluid and saliva). **c)** We will expand these findings to Rapid Influenza Detection Tests (RIDTs).

The expected outcome from this study is the development of a more sensitive diagnostic method that utilizes nanoparticles with a specific bait chemistry tailored to capture, concentrate, and protect Influenza A and B viruses.

# CHAPTER THREE: INFLUENZA

## Section 1: Virology

Influenza (commonly referred to as the "flu") is a serious respiratory illness that is caused by the Influenza virus, a negative-sense RNA virus belonging to the family *Orthomyxoviridae*[5]. This family contains the most segmented genomes of any other family of single-stranded RNA viruses; traditionally consisting of six to eight RNA segments. Unlike most other RNA viruses, both mRNA transcription and genomic replication occurs in the nucleus.

**3.1.1 Genome and Virion Structure and Organization.** The influenza virus, which is approximately 80-120 nm in diameter, consists of a ribonucleoprotein core that is enclosed within a lipid envelope. The influenza particles can be either spherical or filamentous. While most particles are spherical, filamentous particles have been found in lungs of infected individuals. The particles contain roughly 600 spikes protruding from the envelope. These spikes consist of the glycoproteins hemagglutinin (HA) and neuraminidase (NA) in a 5:1 ratio, respectively[5]. The HA protein is responsible for binding to the receptor and mediating membrane fusion, whereas NA mediates the cleavage of the host receptor in the budding stage of the virus' life cycle[5,6]. There is a third envelope protein known as M2. This tetrameric protein is small (97-amino acid) and sparse (20-60 molecules per virus) but plays an important role in the entry and exit of the virus. The M2 protein is an ion channel that is activated by the low pH of endosomes,

leading to disassociation of the matrix protein from the NPs and unpacking of the viral genome[5,7]. The core of the virus is linked to the envelope via the matrix (M1) protein. Within the core, you find the nuclear export protein (NEP), which facilitates the export of progeny genomic RNA segments from the nucleus to the cytoplasm. The core of the virus is comprised of eight helical ribonucleoprotein (vRNP) segments. Each vRNP segment contains four additional types of proteins (PA, PB1, PB2, and NP). NP is the most abundant protein within the vRNP and helps organize the genomic RNA segments into helical vRNPs[5]. The other three proteins – PA, PB1, and PB2 – form the heterotrimeric RNA polymerase complex that is required for the transcription and replication of the virus[8].

**3.1.2 Strains and Subtypes.** The Influenza virus is classified into three types: A, B, and C. Types A and B are transmitted via the respiratory route, but all three types can affect humans. For types A and B, the genome is composed of eight segments that encode ten polypeptides. For type C, there are seven segments. The HA-esterase fusion protein (HEF) of Influenza C caries the three functions of HA and NA proteins of Influenza A and B. Type A accounts for the majority of the seasonal Influenza infections and for all of the pandemics found in human cases[9]. Like most viruses, Influenza A has a high mutation rate, this coupled with its ability to undergo genetic reassortment, leads to variability in the HA and NA glycoproteins[10]. For Influenza A, there are 16 different HA antigens and nine different NA antigens. The combinations based on these two proteins are known as subtypes. The viruses can be further broken down into strains and lineages.

Currently, there are two different lineages for the Influenza B virus – B/ Yamagata and B/Victoria[11,12].

**3.1.2 Influenza Life Cycle.** The virus is transmitted via aerosolization from infected individuals or from fomites. The surface glycoprotein HA of the virus then binds to sialic acid receptors found in the respiratory epithelium. The virus is then taken up by receptor-mediated endocytosis. In order for the release of the vRNPs into the cytosol to occur, HA must be cleaved into two subunits, HA1 and HA2. This occurs when newly synthesized virions are released from the host cells. The low pH within the endosomes causes a conformation change with the HA protein exposing the HA fusion peptide. The fusion peptide of the HA2 subunit allows for the viral and host endosomal membranes to fuse and subsequently release the vRNPs. The released vRNPs are transported from the cytosol to the nucleus, where transcription and replication will occur[5].

As previously mentioned, the Influenza virus is a segmented negative sense RNA virus. The viral RNA (vRNA) serves as a template for the synthesis of both cRNA and mRNA. Transcription occurs early in infection. During this time, vRNA is transcribed into positive sense mRNA by the viral polymerase complex and the NP. A 5'methylated cap must be present for viral transcription to occur. This cap is acquired from the host RNA via a process known as "cap snatching," where endonucleases cleave 10-13 nucleotides from the 5'end of a host mRNA transcript. The nucleotides then serve as primers for viral transcription. The PB1 subunit of the Influenza polymerase complex then binds to a

site at the 5'end of each of the genomic RNA segments to initiate transcription. The mRNAs produced during transcription are prematurely cleaved, and are therefore 15 to 22 nucleotides shorter than vRNA. These transcripts are also polyadenylated; containing approximately 150 A residues at the 3' end of the nascent mRNAs. The mRNAs are then transported back to the cell cytosol with the help of NEP, where translation occurs. Replication occurs later in infection, where vRNA is used as a template to make complementary RNA (cRNA), which is used to make multiple copies of progeny vRNAs. Unlike mRNA in transcription, the cRNA is noncapped, nonpolyadenylated, and full-length positive sense strand[5].

The viral particles are released from the host plasma membrane via budding. Budding is first initiated by the clustering of HA and NA in the lipid raft domains of host plasma membrane[6]. The NA glycoprotein then removes the terminal sialic acid resides found on membrane glycoproteins and allows for the HA proteins of the particles to be released[5].

**Section 2: Epidemiology**

Due to the frequent mutations of the protein coat of Influenza, the virus leads to both seasonal epidemics as well as global pandemics. In the United States alone, the virus is responsible for more than 35,000 deaths and 200,000 hospitalizations each year[13,14]. Several pandemics have occurred during the last century, seen most recently with the appearance of the novel H1N1 strain of Influenza in 2009[15]. While vaccinations against

seasonal Influenza are available, Influenza viruses evolve rapidly and the seasonal Influenza vaccines are oftentimes rendered ineffective[4].

There are two types of genetic variation that can affect the virus' antigenicity – antigenic drift and antigenic shift. Antigenic drift is responsible for the seasonal epidemics. The reason for antigenic drift is because the viral RNA polymerase does not have a proofreading function, allowing for point mutations to occur at a rate of $10^{-4}$ per base per genome replication[5]. These antigenic variants evade the existing antibodies and allow for the virus to evade the immune system numerous times, therefore requiring novel vaccines every year[16]. Antigenic shift is the reason for pandemics throughout the centuries. An antigenic shift, caused from a sudden, major change to the protein structure of HA, is capable of causing global pandemic outbreaks. Antigenic shifts are due to the segmented nature of the viral genome and because of the virus' capability to infect several host species[16]. A human host can be simultaneously infected with a human and a zoonotic Influenza virus, leading to reassortment of the genome segments[11].

Avian and swine influenza viruses are not typically transmitted from birds or swine to humans, nor does their transmission cause disease in humans. However, there have been several pandemics that have risen via mutations and reassortments of avian, swine, and human viruses[17]. The 1918 Spanish flu, which simultaneously infected both humans and swine, was responsible for causing disease in 25-30% of the world's population and causing the deaths of 40-50 million people, many of which were healthy young

adults[18,19]. The Spanish flu was followed by two more pandemics before the turn of the century; the 1957 "Asian" Influenza H2N2 and the 1968 "Hong Kong" Influenza H3N2[19]. The most recent pandemic outbreak has been the H1N1 pandemic of 2009 of swine origin that was first identified in Mexico. Within one month, there were nearly 9000 confirmed cases and 74 deaths from 40 countries. There is significant evolutionary distance between the HA segment of this novel swine-origin virus and its closest subtype relative, demonstrating a lack of surveillance and testing in the swine populations over the years[20,21]. The global spread of swine-origin H1N1 demonstrated how an increase in trade and travel in the last century has substantially increased the risk of transmitting respiratory disease from person-to-person from any part of the world[21].

## Section 3: Factors for Pathogenicity

There are several factors that are responsible for the differences in pathogenicity seen in various strains. An increase in international travel as well as in population crowding are two main reasons why certain strains spread rapidly around the world[21–23]. The virus itself can use the host response to its benefit by eliciting a deadly cytokine storm that leads to viral spread to the lower respiratory system as well as exposure to secondary bacterial infections such as pneumonia[18,24]. The differences in surface glycoproteins HA and NA as well as mutations that can arise in the other viral antigens such as PB1 and PB2 are also responsible for increased disease severity[25–27]. The lack of successful antivirals calls for a better understand on host-pathogen interactions with a focus on conserved viral regions for drug targeting. These findings demonstrate a need for better

surveillance of mutations that occur in animal and human Influenza strains that could lead to another pandemic influenza outbreak.

**3.3.1 Environmental and Social Factors.** Age has long played an important role in the pathogenicity of the virus. Influenza strains cause elevated morbidity and mortality in both children and the elderly[28]. An early study conducted by the Centers of Disease Control (CDC) and Chicago Department of Public Health (CDPH) demonstrated that children ages 5-14 were 14 times more likely to be infected with the 2009 pandemic Influenza A (H1N1) strain[1]. In a 2010 article, it was estimated that acute lower respiratory infections cause one million deaths annually in children under the age of five years[29]. The subtype of Influenza virus appears to also play a role in severity in disease. A 2009 study performed by O'Riordan et al demonstrated that children with pandemic H1N1 infection were significantly older than those with seasonal influenza[28]. This strongly suggests that older children may be protected from seasonal Influenza strains due to pre-existing or cross-reactive immunity to previous seasonal Influenza strains[28]. In the case of seasonal Influenza, infection and severe disease progression is found in the elderly. However, in the case of the 2009 H1N1 pandemic strain, adults older than 60 years of age were disproportionally spared from infection. These finds parallel those of the 1918 Spanish flu, where influenza mortality risk was substantially higher in those less than 65 years of age than those older than 65, and that persons less than 65 years of age accounted for more than 99% of the Influenza-related deaths[19]. These findings are highly suggestive of cross-over immunity from an antigenically similar virus in the past[17].

Seasonal Influenza is most common during the wintertime when there is a drop in temperature. This is supported by findings showing a decreased effect of seasonality in the tropics. The reasoning for this increase in Influenza infection is not entirely known. It is speculated that it may be due to an increase in physiological stress and energy costs for thermoregulation that could weaken the immune system. It could also be due to behavioral changes such as increased crowding. Another theory is the creation of an ideal environment for viral particles; a closed environment with recirculation of a body of air with very low humidity[30]. Since Influenza is a respiratory disease; population growth into urban regions is directly correlated with disease spread. Some scientists believe that the origin of the virus comes from the hypercrowded conditions of military bases during the First World War, and that deployment of the infected soldiers to all regions of the world allowed the spread of the Spanish flu from a localized region to a global front[30]. Respiratory outbreaks, most recently the 2003 SARS outbreak and the 2009 pandemic H1N1 virus, have been directly associated with the increase in international travel for tourism and business by air, ship, and train[22,23].

**3.3.2 Host Factors.** Host susceptibility to Influenza virus is greatly contingent on the number and type of sialic acid receptors found on epithelial cells that are abundant in human upper airways[31]. The series of amino acids found at the cleavage site of host cells have been found to play a role in pathogenicity. Human Influenza viruses recognize and bind to sialic acid linked to galactose by α-2,6-linkages, whereas avian Influenza viruses

recognize α-2,3-linkages[31,32]. Mucin, a heavily glycosylated protein with α-2,3-linked oligosaccharides is thought to bind avian Influenza and prevent infection[31].

Host pathogenicity is highly dependent on the host's innate immune system. While the immune system cannot protect against infection, it can rapidly ameliorate the infection. For example, CD8 T cells elicit a memory response, and will recognize and kill infected host cells[30]. The virus is also recognized by toll-like receptor (TLR) 7 and retinoic acid-inducible gene-I (RIG-I). This in turn activates signaling pathways that lead to type I interferon (IFN) production, NF-kB activation, and subsequent antiviral host responses[33]. Two specific immune responses are important in determining the severity of this respiratory disease – cytokine storm and apoptosis.

Cytokines and chemokines are proteins that promote inflammation during a response to infection[18]. However, infection with both seasonal and pandemic Influenza causes the human host to produce elevated levels of cytokines such as interleukin-6 (IL-6), type I interferons (IFNs), and tumor necrosis factor (TNFα as well as other proinflammatory mediators[34,35]. If prolonged, this cytokine storm (also known as cytokine dysregulation) produces significant tissue damage, and has been found to increase both disease severity and the emergence of pneumonia in Influenza–infected patients[35].

**3.3.3 Viral Factors.** Influenza virus binds via surface glycoproteins NA and HA to sialic acid found in human epithelial cells. This association can cause an extensive range of

disease from fever and cough to pneumonia and encephalitis[9,25]. To date, 16 HA and 9 NA subtypes have been identified[36]. The HA protein plays two important roles: the binding of the virus to host receptors and subsequent host and virus membrane fusion[25]. Past Influenza pandemics of avian origin have shown that the virus contains HA that recognizes human-type receptors. Interestingly, avian-type receptors are found in human lungs, and not in upper respiratory tract cells, shedding light on how the HA glycoprotein plays a role in disease progression to severe pneumonia in humans infected with highly pathogenic avian H5N1 viruses[32]. Furthermore, the HA protein is cleaved by a cellular protease to form HA1 and HA2. The fusion peptide of the HA2 subunit allows for the fusion of the viral and cell membranes. Therefore, virions containing uncleaved HA are not infectious[32,37]. For most Influenza viruses, infection is restricted to the respiratory tract, the only location that the HA proteases such as furin and the transmembrane serine protease TMPRSS2 are produced. There is strong data that suggest that the HA of highly pathogenic avian Influenza can be cleaved by ubiquitous proteases such as TMPRSS4 that are found in lung tissue[37].

Human Influenza viruses do not typically replicate in the lungs. However, in the case of the 1918 pandemic influenza, the virus was capable of efficiently replicating in the lungs (likely due to host cytokine storm damage) and thus causing fatal viral pneumonia[25]. Similarly, there were significantly high viral loads in the lungs of patients infected with 2009 pandemic H1N1 that persisted for up to 17 days[18]. Findings by several scientists support this notion. A contemporary human virus that possessed the pathogenic H1N1

1918 HA glycoprotein replicated at high titers and caused severe lung damage not typically observed with seasonal Influenza infections[25].

Another determinant of virulence is the viral RNA polymerase complex containing the PA, PB1, and PB2, as well as NP. There is strong evidence that points to the role of the viral RNA polymerase complex in the spread of virus to the lower respiratory tract and lungs, leading to pneumonia. A 2008 paper by Watanable et al found high viral titers in the lower respiratory tracts and lungs of ferrets infected with the 1918 pandemic H1N1 or with a reassortant virus containing the 1918 polymerase complex as well as NP. In contrast, substitution of single genes from the 1918 virus did not show these virulence properties[26].

NS1 is another important viral protein that hijacks the host response and controls viral pathogenicity. Influenza virus can escape the antiviral innate immune response via the NS1 protein that interferes with RIG-I signaling pathway. NS1 stops the ubiquitination of RIG-I, which leads to a decrease in type I IFN response. This suppression was seen with the 1918 H1N1 virus by suppressing IFN-regulated genes and therefore controlling antiviral innate immune responses. The latent protein kinase PKR inhibits viral and cellular protein synthesis and virus production by phosphorylating the translation initiation factor eIF2. NS1 evades this system by binding PKR and inhibiting its activation. This in turn prevents the suppression of viral mRNA translation[33,38].

## Section 4: Vaccination

Influenza vaccines against seasonal Influenza are available each year. The vaccine can be administered as a shot or as a nasal spray. Due to the high mutation rate of the virus, there is a need for a reformulation of the vaccine each year. The surface glycoprotein HA and NA are the major targets that are recognized by neutralizing antibodies[5]. The inactivated human Influenza virus used each year is generated from reassortment viruses that contain the HA and NA genomic segments and from strain PR8, a laboratory-adapted avirulent H1N1 strain that contains the six remaining genomic segments[39]. It can be trivalent or quadravalent. Each year, it is comprised of an Influenza H1N1 strain, an H3N2 strain, and one or two Influenza B strains[39].

There is interest in developing a universal Influenza vaccine that would provide protection against all Influenza strains. The primary targets of the universal vaccine have been the M2 protein, which is highly conserved among all Influenza types, as well as the NP, which is a component of the ribonucleprotein complex within the virus. Another developing vaccine target is the conserved region of HA. In 2010, a variation of this vaccine was tested in mice, ferrets and monkeys and produced an immune response to the HA stem, which is another portion of the virus that is highly conserved. Researchers hope the enhanced development of this vaccine will allow for the recognition and neutralization of all Influenza strains[40].

## Section 5: Therapeutics

There are several FDA-approved Influenza antiviral drugs available. Most of the drugs work by targeting the NA and M2 ion channel proteins. The NA protein cleaves the sialic acid receptor from the viral glycoproteins on the host cell surface, thereby releasing progeny virions from the host cell[5,27]. Antiviral drugs against NA (ostelmaivir and zanamivir) bind to the active site of the protein and prevent its enzymatic activity. Unfortunately, there is widespread resistance to the drugs. For example, H1N1 viruses with a NA H274Y mutation are resistant to such drugs[5,32,41,42]. The rate of resistance to certain drugs targeting seasonal Influenza has risen from 0.7% in 2006-2007 to 98.5% in 2008-2009 in the US alone [32]. One explanation for this is the alteration of the hydrophic pocket in the active site of NA that is required for the drug to bind[43]. It is believed that the Oseltamivir-resistant human H1N1 mutant viruses could have emerged in immunocompromised patients that underwent prolonged replication, resulting in increase in fitness of the mutant viruses[32]. Another class of antiviral drugs called Adamantanes block ion channels of the M2 protein, preventing the release of VRNPs into the cytoplasm. However, many human H1N1 viruses have demonstrated resistance to these inhibitors as well[32].

While there are several anti-influenza drugs available, there are important limitations. First, the drugs must be administered during the first 24 or 48 hours after onset of illness. This is oftentimes a critical problem since the first symptoms of the flu are indistinguishable from other respiratory illnesses. Second, while the drugs stop Influenza from replication and spread, it cannot stop the host-induced response to the virus. In

addition, the virus is still highly contagious and the drugs do not stop the spread of the virus from person to person. Lastly, many of the antivirals protect only against certain subtypes. For example, while zanamivir (commonly referred to Relenza) and oseltamivir (commonly referred to as Tamiflu) are affective against most type A and type B virus, amantadine and rimantadine are effective against some (not all) type A influenza virus strains, but not against Influenza B[5,39].

# CHAPTER FOUR: INFLUENZA DIAGNOSTICS

## Section 1: Symptoms.

Symptoms of influenza are similar to those for the common cold. These include a fever of a 100$^{o}$F or higher, cough, sore throat, runny or stuffy nose, headaches, body aches, chills, fatigue, nausea, vomiting, and/or diarrhea. Therefore, misdiagnosis early on in infection is common, which can lead to serious problems later on in infection[39]. It is important to note that individuals are infectious from the day before symptoms occur until up to ten days after symptoms first appear[39,44]. However, several studies have shown a prolonged period of viral shedding of up to several weeks or months in children, elderly, and immunocompromised patients[39].

## Section 2: Clinical specimens.

Respiratory samples for Influenza are obtained from oral fluids, specifically nasopharyngeal or nasal aspirates or swabs. Less frequently, sputum and saliva samples may also be used for diagnostics. Nasopharyngeal aspirates are considered the specimen of choice for the detection of respiratory pathogens. However, this method is more invasive, uncomfortable to the patient, and requires a skilled clinician. Nasal or throat swabs are both safer and easier to use, as well as painless. However, the viral yield is significantly lower for these types of specimens[45,46]. For most accurate results, samples should be obtained during the first four days of illness[47,48].

**Section 3: Serological (Indirect) Testing:**

Serology is the testing of bodily fluids such as a serum, saliva, and urine for specific antibodies against a virus or viral antigens[49]. It is based on the principle of adding viral antigen or host antibody to patient samples. These assays are important in detecting the immunogenicity of vaccines and establishing a diagnosis after a period in which cell culture and PCR testing would yield positive results (patient no longer shedding virus). However, in many cases, antibodies are produced and peak several weeks after infection, making the use of serological tests in clinical settings inefficient[49].

**4.3.1 Hemagglutination inhibition assay.** The HA protein of Influenza agglutinates erythrocyte receptors. The hemagglutination inhibition assay is based on the concept that antibodies against the virus attach to antigenic sites on HA and prevent the binding between HA and receptors on erythrocytes. This test is very reliable and relatively quick that can be utilized in an outbreak scenario (e.g. WHO for global influenza surveillance)[50]. However, there can be nonspecific binding due to non-antibody molecules naturally in the sera as well as agglutinins from other origins. Therefore, standardization must be performed for each test. Furthermore, tests are not quantitative, requiring further analysis with other assays[50].

**4.3.2 Complement Fixation Test.** The complement fixation test is based on the principle that a specific reaction between and antigen and an antibody takes up a "complement" that is taken up by a second detector system of sensitized red cells. A

failure to lyse red cell signals a positive reaction. This method is still commonly used to diagnose "atypical" pneumonia (for example from influenza patients with secondary infections). This technique can detect several pathogens at the same time and is cost-effective. Since this technique is detecting antibodies produced several days to weeks after infection it cannot detect early infection. Another issue that arises with the complement fixation is the lack of sensitivity and cross-reactivity of antigens[49].

**4.3.3 Immunofluorescence.** Immunofluorescence is a technique that uses indicator-labeled antibodies to visualize viral antigens. The indirect method uses a combination of virus-specific antibody and labeled anti-species antibody[49] that is detected using a light microscope. This method is more sensitive compared to other methods. It is also quick, and results can be obtained within one to two hours. However, some of the disadvantages of this technique are that it is dependent on high-quality specimen and rapid processing of the samples to avoid reduction in sensitivity. Furthermore, the interpretation is highly subjective and not definitive[49].

**4.3.4 Enzyme-Linked Immunosorbent Assays.** ELISAs are used to detect the viral antigens such as the NP or antibodies such as IgG or IgM. There are several types of ELISAs: indirect, sandwich, and competitive. Some advantages of ELISAs are that they are a good indicator of recent (via capture IgM assay) or of prior infection (via IgG assay). Furthermore, they can be used to detect uncultivable or poorly cultivable virus, such as Hepatitis B Virus[49]. ELISAs allow for the rapid detection of viral antigens or

antibodies against a virus, but the technique is cumbersome, expensive, and may pose a risk to laboratory personnel[51,52]. Furthermore, several washing steps make this assay tedious and time-consuming. Sandwich ELISAs for antigen detection (sAG-ELISAs) provide a safer and faster detection method[52]. The specimens are first heat and detergent inactivated before testing. However, viral titers typically remain high for a few days and may reach undetectable concentrations at both early and late time points post-infection[51].

**4.3.5 Rapid Influenza Diagnostic Tests (RIDTs).** RIDTs are lateral flow assays derived from antigen ELISA methodology. The assay works by disrupting the viral particles with an elution buffer and exposing viral NPs. If present, the viral antigens will bind monoclonal antibody reagents that are specific to Influenza[53]. They can identify the presence of the Influenza A or B viral NP in respiratory samples in 15 minutes or less. RIDTs are a useful screening tool that provides a qualitative (not quantitative) answer. However, one of the limitations of the RIDTs is their limited sensitively at low concentrations, which can lead to high rates of false negative results. Studies conducted by CDC have shown that RIDTs are only 50-70% sensitive when compared to qRT-PCR analysis, leading to many false negative results[47]. A more recent study by Peci et al demonstrated that RIDTs had a low sensitivity of 60% for type A viruses and a very low sensitivity of less than 38% for type B viruses[54]. This is largely due to the use of only a small volume of sample in these assays as well as the inclusion and subsequent interference of high abundant proteins in complex solutions such as nasopharyngeal aspirates and swab samples. Moreover, the virus is highly degradable and samples

require transport at 4°C or lower. Furthermore, the tests are highly specific and results varied based on virus type, subtype, and strain[55,56]. For example, sensitivity for the 2009 H1N1 influenza A subtype is significantly lower than the sensitivity for seasonal influenza[56]. Therefore, specimens must be collected as early in the illness as possible (by 72 hours after infection) when viral levels are high and a definitive diagnosis must be confirmed by molecular assays or viral culture[57].

**Section 4: Direct Testing:**

Direct methodology includes virus isolation, genome detection, and antigen detection.

**4.4.1 Virus isolation.** Virus isolation has long been considered the gold standard for viral diagnosis. In the past viral culturing required propagation in laboratory animals or embryonated eggs, however, most virus-isolation techniques now occur in cultured cell lines[48,49]. Infectious virus can be detected by cytopathic effect (CPE) in cell lines such as Madine-Darby canine kidney cells. However, since various respiratory pathogens can cause CPE in one cell line, other methodologies are needed to confirm an Influenza diagnosis. These include haemadsorption assays and complement fixation. This in turn allows for the comparison of novel, circulating, and vaccine strains of the virus and plays an important role in treatment and antiviral resistance[6].

There may be a limited number of clinical samples provided per human case. Therefore it is important to reculture the pathogen so that it can be utilized for future experiments.

In order to preserve the integrity of the virus, samples for viral culturing must be kept on dry ice or frozen at a temperature of at least -70$^{o}$C immediately after collection to prevent decreased viral titers[58]. While RIDTs and PCR provide results within minutes and hours, respectively, results from viral culturing takes least 3-14 days[39,59,60]. These conditions are not optimal when facing a potential epidemic or in clinical settings when rapid diagnosis is needed[49,51,56,61]. Therefore, there is a substantial need for accurate and reliable sample preparation methodology that concentrates Influenza from clinically complex specimens, protects the virus from degradation over time and elevated temperatures, and can be coupled to various diagnostic assays for Influenza.

**4.4.2 Electron Microscopy.** Electron microscopy is the only technique that allows for direct visualization of the virus. It uses an electron beam to dramatically magnify the specimen up to 10,000,000X. The advantages of electron microscopy include the detection of unculturable viruses and rapid detection. However, it is not the best method in clinical settings due to its poor sensitivity when viral titers may be low in clinical samples[49] .

**4.4.3 Molecular assays.** As previously mentioned, global health organizations such as the WHO, the CDC, and the Infectious Disease Society of American (ISDA) highly recommend confirmation of RIDT results with a molecular methodology. Molecular assays such as RT-PCR and qRT-PCR are able to identify viral RNA, most commonly from the M1 gene, from various strains and subtypes of Influenza[39,62]. RT-PCR is the

preferred testing method because it is the most sensitive and specific methodology, and results can be obtained within hours[39]. Furthermore, the development of multiplex PCR allows for the amplification of more than one respiratory viral target in the same test, allowing for more accurate diagnoses. However, one limitation is that many assays do not distinguish between the varying subtypes or strains of the Influenza virus[4]. Another limitation is that the sample must be kept at a temperature of at least 4$^{o}$C in order to preserve the integrity of the viral genome[58]. Additionally, PCR detects both infectious and noninfectious RNA. Therefore, detection of viral RNA by these assays is not indicative of viable virus or on-going Influenza viral replication in the respiratory specimen and this is only possible with viral culturing[6].

**Section 5: Challenges of Influenza Detection.**

There are numerous circulating and novel subtypes and strains of Influenza virus that continuously pose a risk to both livestock and human populations. The handling of the novel strains of virus is a risk to laboratory personnel, with highly pathogenic avian influenza strains requiring handling in Biosafety Level 3 facilities with highly trained personnel[63]. There is a necessity for an upstream methodology that can inactivate the potential pathogen without compromising detection with molecular or antigen-based assays.

Another issue is viral titer. During Influenza infection, the virus exponentially grows and peaks at 2 to 3 days post infection. The virus is undetectable after 6 to 8 days post

infection[64]. Based on the viral growth kinetics, conventional diagnostic methods have a very limited timeframe for detection and are most accurate early in disease progression. Several serological methods recognize antibodies against the virus later in infections when the damage is done and secondary complications are likely to occur. Furthermore, the high rate of degradation seen with Influenza viruses does not allow viral transfer in ambient conditions without sacrificing viral titer. Therefore, there is a need for a technology that will concentrate virus at low viral titers while simultaneously preserving viral titers.

## Section 6: Novel and Future Diagnostic Methodologies.

Any novel technology must be time and cost-efficient, as well as reliable yielding low false-negative and false-positive results. There are several promising technologies available that can potentially answer all the diagnostic problems for Influenza.

**4.6.1 Pan-viral microarray assay.** Microarrays are a fairly recent technology that was developed in 1992 from the Southern blotting technique. It consists of a chip (such as a microscopic glass slide) that contains up to hundreds of thousands of probes that are specific DNA sequences[65]. In the case of RNA viruses, the RNA is first extracted, reverse transcribed to cDNA, and labeled with a fluorescent dye. The sample is mixed with a hybridization solution and added onto the microarray. The samples are allowed to hybridize for several hours before the sample is washed to remove any nonspecific binding. Total signal strength of fully and partially complementary strands is then

measured. One advantage/disadvantage lies in oligonucleotide (oligo) size. Long oligos have higher sensitivity, whereas short oligos have higher selectivity and can better recognize unspecific hybridization. Another disadvantage is the risk of cross-contamination of probes.

One recent advantage of microarrays is the capability to simultaneously detect hundreds of pathogens on one chip with the development of a Pan-Viral Microarray Assay (ViroChip). This is done with the use of various probes that are specific for different pathogens. Scientists have used this methodology in the last few years to successfully identify the full spectrum of viruses that are associated with respiratory infections as well as identify novel viruses such as the SARS coronavirus. While this is a very promising technique in pathogen discovery, the methodology is complex, meticulous, and lengthy, and therefore requires highly trained technicians[66].

**4.6.2 Ligation detection reaction-universal array.** The ligation detection reaction combined with universal arrays (LDR-UA) uses two probes; one probe that is fluorescently labeled (known as the discriminating probe) and a second probe (known as the common probe) that contains a "Zip Code" sequence that is unique for each probe set. The two probes anneal juxtaposed to the target site of the sample DNA. Perfect complementarity between the 3′ end of the discriminating probe and the target DNA allows for the two molecules to ligate together[67]. The ligated product is then hybridized to the UA, which is an oligonucleotide DNA microarray that contains the complementary

sequence of the "Zip Code" (known as cZC). Therefore, the common probe binds the cZC and the array spot will fluoresce. This method is more cost-effective, highly sensitive, and more flexible for the detection of pathogens compared to cDNA microarrays. However, the method is still slow compared to the TaqMan PCR methodology[68].

**4.6.3 AlphaLISA Immunoassay.** A no-wash alternative to ELISAs is the novel AlphaLISA immunoassay, which can be used for the detection of analytes in various biological samples. Like ELISAs they require the use of antibodies, but require no washing steps and can yield results in less than two hours[69]. It is based on a "sandwich" formation consisting of capture antibody-analyte-reporter-antibody. When an analyte is present, the donor antibody comes into close proximity with the acceptor and a signal is observed. This is due to acceptor emission upon donor excitation based on the Forester Resonance Energy Transfer (FRET) mechanism[70]. This methodology was most recently used in 2013 in the detection of *B. anthracis* spores and protective antigen (PA) toxin in the sera of anthrax-infected rabbits[70].

**4.6.4 Luminex Assay.** Another fairly recent and developing methodology is the Luminex assay. Beads with fluorescent dyes are conjugated to biomarkers such as antibodies. They can capture and detect specific analytes from a complex samples such a serum, urine, and saliva. Up to 500 proteins or genes can be detected from a very small sample volume. This is especially important in clinical samples where volumes are

limited. There are several other advantages of the Luminex assay over other assays, including high sensitivity, throughput, and efficiency. When compared to expensive assays such as ELISAs, there is a significant reduction in time, labor, and cost[71,72]. Luminex sensitivity is significantly higher and limit of detection is three times lower compared to ELISAs[71]. The disadvantages include possible cross-reactivity between antibodies and compromised sensitivity as the number of different beads increase. This developing assay has been used in a variety of different diseases, from cancer to bacterial and viral infections. The assay was successfully used to detect serum IgG concentrations from the sera of *Streptococcus pneumonia* patients[71]. It has also been used to detect oral cancer biomarkers in saliva (specifically increased expression of IL-8 and IL-1B), demonstrating the important role of this assay as a noninvasive tool in early disease detection[72]. The assay has been extended to respiratory virus screening and detection. A study conducted by Pabbaraju et al in 2008 demonstrated that a higher number of respiratory specimens tested positive for one of 20 respiratory targets with the Luminex assay (690) compared to multiplex PCR (643 samples)[73]. While PCR methods are now highly sensitive and specific, Luminex assays provide the advantage of using more beads for pathogen detection, making it ideal for complex analysis of respiratory virus detection[73].

**4.6.5 Loop-Mediated Isothermal Amplification.** The loop-mediated isothermal amplification (LAMP), which allows for one-step amplification of reactions under isothermal reactions[48,74]. This molecular technique is similar to PCR and involves

primers and DNA polymerases[75]. The results can be detected visually by fluorescence strength or the metalochrome indicator calcein[75]. While this diagnostic method has been available since 2000, a paper published by Katano et al in 2009 demonstrates that LAMP can be coupled to a disposable pocket warmer as the heat block for the detection of anthrax. Surprisingly, this inexpensive tool is the only equipment (besides reagents and samples) that is necessary for the assay[74].

**4.6.6 Nanotechnology**. Nanotechnology is a developing area of interest in the detection of infectious diseases. Several scientists have worked together to develop a network of gold particles linked together to multiple DNA strands that are in combination with DNAzyme (an enzyme that is made completely of DNA). In the presence of a particular infectious pathogen, the DNAzyme is activated and cleaves the DNA links holding the nanoparticles together. When the pathogen is present a red color is produced (dispersed nanoparticles) whereas if the pathogen is absent a purple color remains. This technology has been utilized to detect novel dengue virus, hepatitis B virus, and malarial parasites and can easily be extended to the other viruses[76,77].

# CHAPTER FIVE: NANOTRAP PARTICLES

## Section 1: Nanotrap particle technology

Nanotrap particles are customizable hydrogel microspheres developed by Ceres Nanosciences for target analyte separation and discovery applications. These particles have demonstrated their utility as innovative tools for the collection, concentration, and preservation of dilute levels of low molecular weight peptides, proteins, and other biomolecules from biofluid samples[78]. Nanotrap particles are based on cross linked N isopropylacrylamide (NIPAm) and are appealing as analyte sequestration and concentration devices due to their versatility, reproducibility, and low production costs[2,3,78–80]. The monomers used to generate the Nanotrap particles also have good colloidal stability in biofluids, thereby creating a large surface area that is ideal for the rapid and complete capture of target analytes in complex aqueous biological matrices[2]. The cross linked polymeric networks that make up the Nanotrap particles are highly hydrated, making it possible for small molecules to attain access to the interior of the particle[80]. Furthermore, the thermoresponsive nature of the NIPAm monomer imparts on the particles, a significant degree of flexibility and porosity in response to changes in pH and temperature[80]. Thermoresponsive hydrogels have been extensively studied for drug delivery applications, and the knowledge attained from these studies can be applied toward engineering Nanotrap particles for analyte collection devices[79].

Nanotrap particles can be functionalized with a variety of affinity baits to facilitate the binding and retention of collected target proteins, peptides, post translationally modified analytes, lipids and fatty acids, metabolites, nucleic acids, and pathogens[4,81]. This versatile nature of these Nanotrap particles also allows them to be tailored to capture target analytes from a variety of complex biological matrices, including blood, serum, plasma, saliva, and nasopharyngeal fluids[82,83]. One method of functionalizing the particles involves polymerizing the NIPAm monomer with another monomer species to generate a copolymer hydrogel possessing the chemical properties of both monomers. For example, poly NIPAm (p NIPAm) particles with incorporated acrylic acid (AAc) moieties were synthesized via precipitation polymerization to create negatively charged thermoresponsive Nanotrap particles. These particles demonstrated the ability to harvest and concentrate low molecular weight protein species from serum[3]. Another way of functionalizing the particles is to utilize the reversible broad-spectrum protein binding ability of reactive dyes, which are commonly used as ligands for affinity chromatography applications. These reactive dyes can be covalently immobilized onto the polymer matrix, and their low cost and wide commercial availability make them appealing affinity baits for the Nanotrap particles. The Nanotrap particle can also be encapsulated within a cross-linked p NIPAm shell to further increase the sieving functionality of the core particle architecture. This cross linked outer shell of the particle can either be inert or contain chemical moieties such as vinyl sulfonic acid (VSA), which has demonstrated the ability to actively exclude interfering albumin peptides of all sizes while simultaneously excluding large unwanted abundant molecules such as immunoglobulins[4]. The inverse

effect can also be achieved by varying the degree of cross-linking in the particles. While Nanotrap particles with a high degree of cross linking are compact with decreased thermoresponsiveness, particles with low amounts of cross linking exhibit a greater degree of volume phase transition in response to changes in temperature. Elimination of cross linker from aspects of the particle architecture can also tailor the particles for the capture of larger analytes, like virus particles. For example, interpenetrating polymer networks (IPNs) can be included into the particle microsphere structure to introduce environmentally responsive linear or branched polymer chains to the particle[84]. Another method is to synthesize a cross linker free shell structure around the core particles to generate a particle with a shell capable of expanding without the pore size limiting effect of the cross linker[85].

**Section 2: Nanotrap particles and their role in viral detection**

While the Nanotrap particles had originally been designed to specifically harvest proteins and other small molecules, recent findings have shown that the Nanotrap particles can also be used in the capture and detection of whole virus. A recent paper published by Shafagati et al in 2013 demonstrated the capture and enrichment of Rift Valley Fever Virus (RVFV) virions at both high and low viral titers[82]. The virus is further protected from degradation at both increased timepoints (up to 72 hours) and elevated temperatures (up to 37$^{o}$C). Moreover, the virus may be fully inactivated by heat or detergent yet remains bound and therefore detectable by downstream methodologies[82]. The ability of the Nanotrap particles to capture virions allows samples to be analyzed through numerous

downstream analytical techniques (both protein and nucleic acid based) including standard sandwich ELISAs, lateral flow immunoassays, Mass Spectroscopy (MS) techniques, or qRT-PCR assays. These findings with RVFV have been expanded to other viruses such as Human Immunodeficiency Virus (HIV) and Venezuelan Equine Encephalitis Virus (VEEV)[83,86].

Here data is presented indicating that the Nanotrap particles can also bind and concentrate Influenza viruses. It was hypothesized that these Nanotrap particles will be able to not only enrich the virus, but also protect it from degradation over time and at increased temperatures. The data demonstrates the coupling of this novel sample preparation tool to plaque assays, qRT-PCR, western blotting, as well as lateral flow assays, to enhance viral detection at low concentration. Lastly, it is shown that Nanotrap capture is not curtailed in a coinfection scenario with various subtypes and strains of Influenza as well as other respiratory viruses and bacteria.

## CHAPTER SIX: METHODS AND MATERIALS

**Nanotrap particles.** The NIPAm/AA Nanotrap particles were provided by Ceres Nanosciences, Manassas, VA.

**Cell culture.** The Madine-Darby canine kidney (MDCK) cell line was obtained from American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% glutamax (DMEM+++) and cultured in a humidified environment containing 5% $CO_2$ at 37°C.

**Viruses and Bacteria.** Influenza A/California/04/09, Influenza B/Taiwan/2/62, Influenza A/Brisbane/10/2007, Influenza A/Brisbane/59/2007, Influenza A/Texas/62/2009, Human Coronavirus (229E strain) were obtained from BEI Resources, and *Streptococcus pneumoniae* (SPEC1 strain) were obtained from Influenza Resource Repository. Influenza viruses were propagated by infecting 80-90% confluent MDCK cells at an MOI of 0.1 in Influenza Growth Media (IGM; DMEM supplemented with 1% bovine serum albumin, 1% non-essential amino acids, 1% L-glutamine, and 1% penicillin/streptomycin). Cell culture medium was collected from the cells when ~80% cytopathic effect was observed (typically 72 hours post-infection (hpi)). Cell culture medium was centrifuged at 10,000 rpm for 10 minutes to pellet the cellular debris. Cell

free-viral supernatants were then filtered using a 0.22 µM filter and viral titer determined by plaque assays.

**Cell lysates.** MDCK were infected Influenza A/Brisbane/10/2007 and collected 24 hours post-infection. Cell free viral supernatants were removed and the cells were washed one time with Phosphate Buffered Saline (PBS). Cells were trypsinized and washed with PBS. The cells were then centrifuged at 1800 rpm for five minutes. The PBS was removed and the cells were lysed using a clear lysis buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM $Na_3VO_4$. The concentration of the protein was obtained using Bradford reagent.

**Influenza NP.** Recombinant NP with Histidine Tag, from Influenza A/Brisbane/10/2007 (H3N2) was obtained from Influenza Reagent Repository.

**Specimen samples.** Nasal fluid, from a single human donor, was obtained from Lee Biosolutions (991-13-5). Human saliva was obtained from BioreclamationIVT (HMSALIVA). The transport media obtained from BD Universal viral transport kits (Fisher Scientific) were used for the ambient and elevated stability experiments. Samples were sonicated using the MISONIX Ultrasonic Liquid Processor (XL-2000 Series model) for six ten-second pulses at 600 watts. Between pulses, the samples were incubated on ice for 20 seconds. Nasal swabs obtained from BinaxNOW® Influenza A and B kits were used in the mock-swab scenarios.

**Standard Nanotrap particle incubation.** According to a protocol standardized by Ceres Nanosciences, 1000 microliters (µL) of sample (containing virus spiked in either IGM or PBS) was incubated with 100 µL of Nanotrap particles for 30 minutes at room temperature. The sample was centrifuged at 10,000 rpm for 5 minutes and the supernatant discarded. The pellet was then resuspended in the appropriate buffer for downstream analysis.

**Plaque assays.** MDCK cells were plated in 6 well plates at 1.0E+06 cells/ml in order to achieve 100% confluency. After Nanotrap particle incubation, the pellet containing captured viruses was resuspended in 100 µL of IGM and serial dilutions performed. Four hundred µL of each serial dilution was added to each well in duplicate and incubated for 50 minutes. The primary overlay (known as the CV mixture) consisted of equal parts 0.9% agarose in distilled water and media containing 2X EMEM, 5% BSA, 2% non-essential amino acids, 2% penicillin/streptomycin, 2% sodium pyruvate, and 2% L-glutamine. The CV mixture was treated with 0.011% of 2 mg/ml Trypsin-TPCK (obtained from Sigma-Aldrich). Three hundred milliliters (ml) of the CV mixture was added directly to each well and the plates incubated at 37°C. The cells were fixed with 10% formaldehyde in water after 48-72 hpi. The cells were stained with 1% Crystal Violet in 20% ethanol and water. After two hours, the crystal violet stain was washed off and the plaques formed were counted to determine the plaque forming units per milliliter (pfu/ml).

**MagMax RNA extraction and quantitative real time PCR.** After Nanotrap particle incubation, the pellet was resuspended in 130 µL of lysis/binding solution (Life Technologies) containing guanidinium thiocyanate and incubated on ice for thirty minutes. The samples were spun at 13,000 rpm for 5 minutes at room temperature. The supernatant was transferred to a 96-well plate and RNA extraction was performed with Ambion's MagMax 96-well Viral RNA extraction kit according to manufacturer's instructions. In order to determine the number of viral genomic copies produced, qRT-PCR with viral specific primers was performed using RNA UltraSense One-Step Quantitative RT-PCR System (Life Technologies). The experiment was performed according to a standardized protocol using 20µL of master mix containing enzyme mix, 5X reaction mix, ROX reference dye, 10 µM TaqMan fluorogenic probe, 40 µM forward primer and 40 uM reverse primer added to 5µL of extracted RNA. The primer and probe set was obtained from BEI resources (NR-15592 and NR-15592) and recognized Influenza A or Influenza B-specific genes. The thermocycle programs was as follows: 50°C for 30 minutes, 95°C for 2 minutes, and 45 cycles of 95°C (15 minutes) and 55°C (30 minutes).

**cDNA synthesis and PCR.** RNA was extracted according to a standardized protocol for Trizol® LS reagent. cDNA was synthesized using the high capacity RNA-to-cDNA kit (Applied Biosystems). For PCR, a combination of 22.5µl of Platinum PCR Supermix (Life Technologies), 0.2 uM of primer mix (Influenza A matrix protein reverse primer: 5'-CAA-AGC-GTC-TAC-GCT-GCA-GTC-C-3', Influenza A matrix forward primer: 5'-AAG-ACC-AAT-CCT-GTC-ACC-TCT-GA-3', Influenza B matrix protein reverse primer: 5'-TTC-TTT-CCC-ACC-GAA-CCA-AC-3', Influenza matrix protein forward primer: 5'-GAG-ACA-CAA-TTG-CCT-ACC-TGC-TT-3'), and 1µl sample were placed into each tube and the reaction was run using the following temperature and times: 94$^{o}$C for 2 minutes, followed by 35 cycles with 94$^{o}$C for 15 seconds and with 55$^{o}$C for 15 seconds for Influenza A or 53$^{o}$C for 15 seconds for Influenza B, followed by 72$^{o}$C for 10 minutes. Products were visualized using gel electrophoresis. Ten microliters of sample were diluted with 1µl of blue/orange loading dye (Promega) and run on a gel containing two percent agarose diluted in TAE buffer and stained with 0.5ug/ml ethidium bromide. The samples were run at 100 volts for approximately 45 minutes in 1X TAE buffer, followed by visualization under UV light.

**Western Blot Analysis.** Nanotrap pellets containing captured virus and/or NP were resuspended in 25 µL of blue lysis buffer (containing 1:1 mixture of T-PER reagent (Pierce, IL), 2× Tris-glycine SDS sample buffer (Novex, Invitrogen), 33 mM DTT, and protease and phosphatase inhibitor cocktail (1× Halt cocktail, Pierce)). No Nanotrap control samples were resuspended in 10 µL of lysis buffer. All samples were boiled for 10 min. The Nanotrap samples were then centrifuged at 14,000 rpm, the supernatant

saved and samples centrifuged for a second time to ensure that all Nanotrap particles were pelleted. The supernatants were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose or PVDF membranes at 80 mA at 4°C overnight. The membranes were blocked with 3% bovine serum albumin (BSA) in 1× PBS +0.1% Tween (PBS-T) for 1 hour at room temperature. The primary antibodies were diluted in 3% milk in PBS-T at a 1:4000 dilution for the NP antibody. The membranes were then washed 3 times with PBS-T and incubated with secondary HRP-coupled anti-mouse antibody diluted 1:1,000 in 3% milk for 2 hours and then washed 4 times with PBS-T for 5 minutes. The mouse monoclonal antibody to recombinant NP from Influenza A/Brisbane/10/2007 (H3N2) was obtained from Influenza Reagent Repository. The western blots were visualized by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate kit (ThermoScientific) and a Bio-Rad Molecular Imager ChemiDoc XRS system (Bio-Rad). The band analysis tools of Quantity One 1-D analysis software (Bio-Rad) were used to determine the background-subtracted density of the bands in the western blots. Fold enrichment was determined by dividing the (+)NT samples by the (-)NT control samples.

**Lateral Flow Assay.** Alere BinaxNOW® Influenza A and B kits were used for the lateral flow assays. The following elution buffers were used: elution buffer from the Alere BinaxNOW® Kit, clear lysis buffer, blue lysis buffer, T-PER reagent (Life Technologies), and 1M imidazole dissolved in distilled water.

**Control and -NT Samples.** Control samples used throughout the paper consisted of 10 µl of sample (containing no Nanotrap particles) added to 10µl blue lysis buffer. Samples not incubated with Nanotrap particles (-NT samples) consisted of 10 µl of the sample incubated at room temperature for 30 minutes (in parallel with Nanotrap particle-incubated samples) and then added to 10µl blue lysis buffer. Both the control and -NT samples were boiled and centrifuged in parallel with +NT samples.

# CHAPTER SEVEN: RESULTS

## Section 1: Nanotrap particles capture Influenza A virus.

In order to determine if Nanotrap particles were able to capture Influenza A, five Nanotrap particles (NT45, NT46, NT53, NT55, and NT69) representing five different bait chemistries were incubated with 100µl of Influenza A/California/04/09 (Table 1; Figure 1A). RNA was extracted and subjected to RT-PCR analysis PCR products were separated by gel electrophoresis and visualized using ethidium bromide staining. The results demonstrated that NT55, an acrylic acid core particle containing no outer shell, and NT69, a cibacron yellow core-shell particle containing an outer VSA shell, were the top two candidates for capture of Influenza A. In contrast, NT46, a reactive red core particle, was unable to capture any virus or viral RNA. We next performed a plaque assay with our top two Nanotrap particle candidates to ensure that infectious virions and not free floating or RNA from a lysed cell that is being captured. NT55 was able to capture, and most importantly, enrich virions (Figure 1B). NT69 provided only a slight (1.5-fold) enrichment compared to control (no NT) samples. From these two experiments, we concluded that an acrylic acid particle is optimal for capturing Influenza A.

**Table 1: Nanotrap particles used in initial screenings**

| Nanotrap ID | Bait | Shell (y/n) | VSA* shell (y/n) |
|---|---|---|---|
| NT45 | Reactive red 120 + Reactive yellow 86 | No | N/A |
| NT46 | Reactive red 120 | No | N/A |
| NT53 | Cibacron blue F3GA | Yes | No |
| NT55 | Acrylic Acid | Yes | No |
| NT69 | Cibacron Yellow 3GP | Yes | Yes |

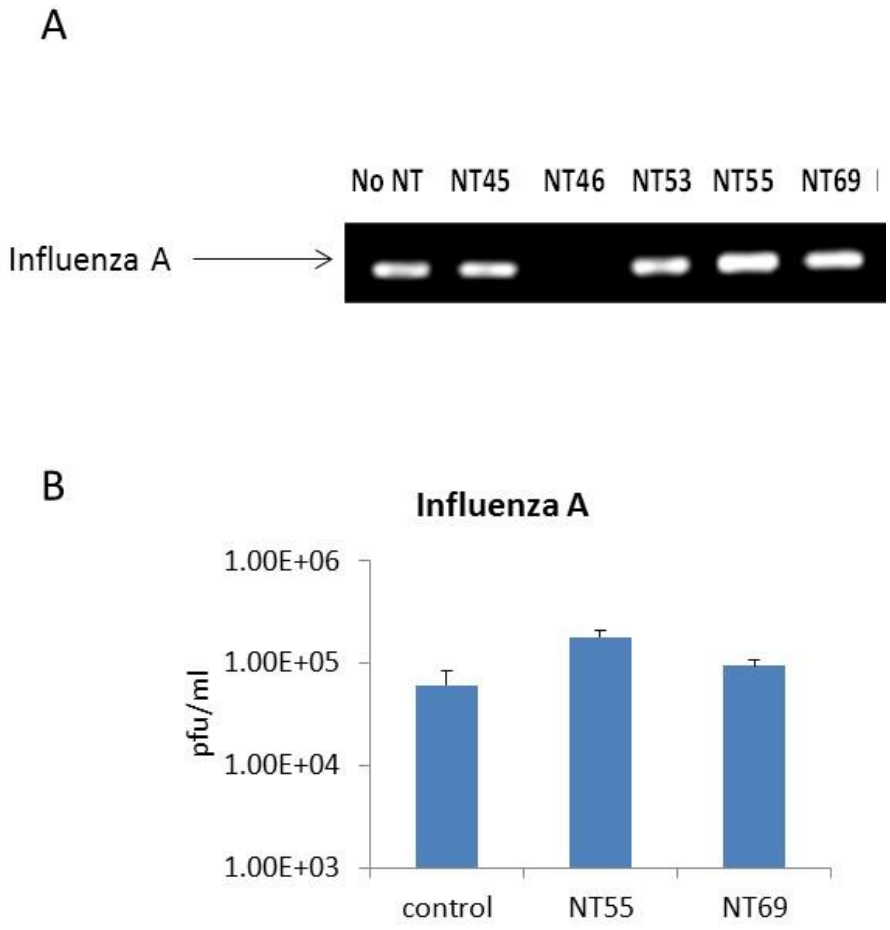


**Figure 1: Nanotrap particles capture Influenza A.** A) One hundred microliters of viral supernatants were incubated with 75 µL of each Nanotrap particle for 30 minutes at room temperature. Viral RNA was extracted and amplified with viral specific primers using either RT-PCR. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. No Nanotrap particle control is at 10µl volume. B) One milliliter of viral supernatant was incubated with 100µl of each Nanotrap particle for 30 minutes at room temperature. After centrifugation, the pellets were resuspended in media (supplemented DMEM). Serial dilutions and subsequent plaque assays were performed. After 72 hours, wells were fixed and stained with crystal violet. Control samples with no Nanotrap particles were processed in parallel.

**Section 2: Acrylic acid Nanotrap particles enhance viral detection.**

Since we had determined that NT55 is a good candidate for virion capture, we wanted to expand upon these findings and look at other acrylic acid particles with varying surface area and different acrylic acid bait conditions. We therefore tested three other acrylic acid particles with different bait and core-shell chemistries (Table 2). We found that NT120 was the top enricher of virion (as demonstrated by plaque assays), providing a 5-fold enrichment best enrichment of 5-fold (Figure 2). NT120 is a core (no shell) particle that is roughly 600 nm in diameter with a bait that consists of 10% methylacrylate saponified to acrylic acid. We therefore focused our subsequent experiments on evaluating the acrylic acid NT120 particle for Influenza capture.

**Table 2: Acrylic acid Nanotrap particles**

| Nanotrap ID | Shell | Architecture | non-crosslinked interpenetrating network 'shell' surface ("arms") | Diameter |
|---|---|---|---|---|
| NT36 | No | 10% acrylic acid | No | 1127 |
| NT55 | Yes | 10% acrylic acid | No | 800 |
| NT120 | No | 15% methylacrylate saponified to acrylic acid | No | 590 |
| NT156 | No | 15% methylacrylate saponified to acrylic acid | Yes | 3178 |

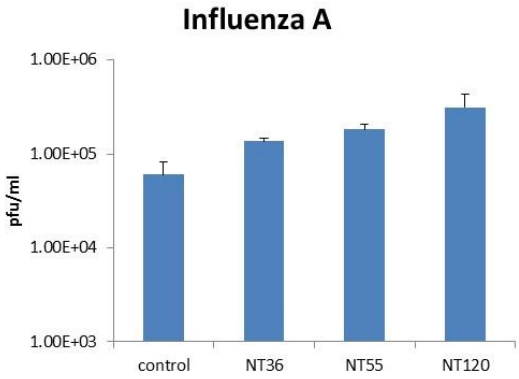


**Figure 2: Acrylic acid Nanotrap particles enrich Influenza A.** One milliliter of viral supernatant was incubated with 100µl of each acrylic acid Nanotrap particle for 30 minutes at room temperature. After centrifugation, the pellets were resuspended in media (supplemented DMEM). Serial dilutions and subsequent plaque assays were performed. After 72 hours, wells were fixed and stained with crystal violet. Control samples with no Nanotrap particles were processed in parallel.

We confirmed the enrichment capability of NT120 in three other strains of Influenza A – Influenza A/Texas, Influenza A/Brisbane/10 and Influenza A/Brisbane/59. Enrichment with NT120 was lower in these three subtypes, at around 2-3-fold enrichment with both plaque assays and qRT-PCR (Figure 3A and B). We hypothesized that Nanotrap particles could be utilized for the capture of Influenza viral particles by interacting and binding to the viral glycoproteins, hemagglutinin and neuraminidase. Therefore, since the HA and NA glycoproteins differ in each subtype and strain, there may be other Nanotrap particles that bind more efficiently to each subtype's and strain's glycoproteins.

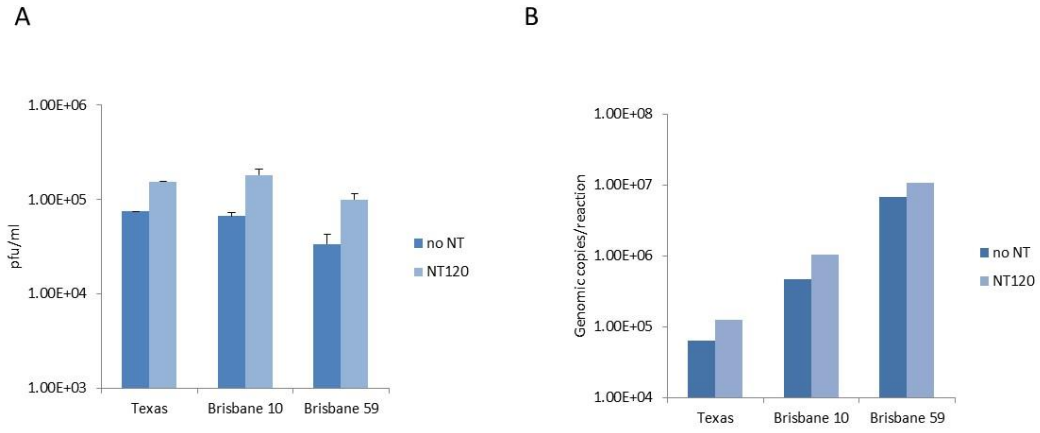


**Figure 3: NT120 captures other Influenza A subtypes and strains.** One milliliter of Influenza A/Texas, Influenza A/Brisbane/10, or Influenza A/Brisbane/59 viral supernatant was incubated with 100µl of NT120 for 30 minutes at room temperature. Samples were centrifuged and unbound material was removed. A) The pellets were resuspended in media (supplemented DMEM). Serial dilutions and subsequent plaque assays were performed. After 72 hours, wells were fixed and stained with crystal violet. Control samples with no Nanotrap particles were processed in parallel. B) The pellets were resuspended in Ambion's MagMax viral RNA lysis binding solution and RNA extraction was performed using the Ambion's MaxMax viral RNA extraction kit. Following RNA extraction, qRT-PCR with viral specific primers specific to Influenza A was performed. Control samples with no Nanotrap particles were processed in parallel.

The Nanotrap particles are processed to be the same mass. However, there may be different individual particle amounts depending on the mass of each type of particle. We hypothesized that the relatively small size of NT120 may be allowing for less competition of viral particles with each Nanotrap particle. Therefore, we tested a significantly larger particle, NT156, which is more than 3000 nm in diameter and contains non-cross-linked interpenetrating network surface that creates "arm" structures

protruding from the Nanotrap particle core (Table 2). We hypothesized that more viral particles would be allowed to stick on one single NT156 particle. We tested both Influenza A H1N1 strain and Influenza B, both of which are included in each year's Influenza vaccine. While Influenza B infections are not as common or severe as Influenza A infections, there are still reports of several fatal cases, especially in the pediatric population[39]. Our plaque assay results demonstrated that both NT120 and NT156 provided a 6-fold enrichment in detection for both Influenza A and Influenza B (Figure 4A and B). Our qRT-PCR results demonstrated a 3-4-fold increase in detection with both particles, with NT156 performing slightly better for both Influenza A and Influenza B (Figure 4C and D). We concluded that both NT120 and NT156 have good enrichment capabilities and therefore utilized both particles in subsequent experiments.

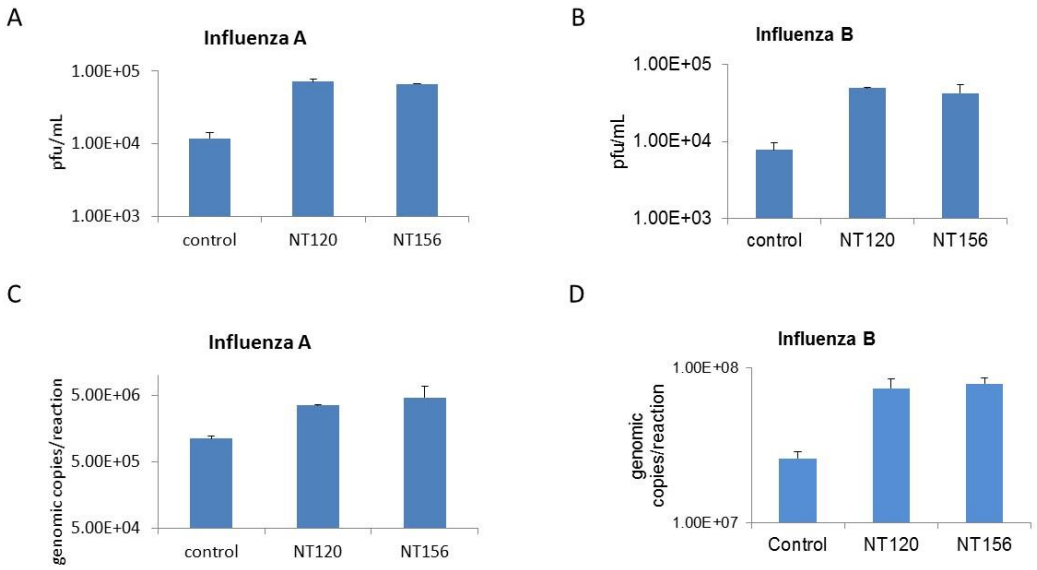


**Figure 4: Acrylic acid Nanotrap particles can capture virions.** Nanotrap particles with acrylic acid baits were incubated with Influenza A or Influenza B diluted in PBS to 1E+04 pfu/ml for 30 minutes. Samples were spun and unbound supernatant was removed. For panels A and B, Influenza A (A) and Influenza B (B) samples were resuspended in Ambion's MaxMax viral RNA lysis/binding solution followed by RNA extraction with the Ambion's MaxMax viral RNA extraction kit. QRT-PCR assays using Influenza A-specific or Influenza B-specific primers and probes were subsequently performed. For panels C and D, Influenza A (C) and Influenza B (D) samples were resuspended in Influenza Growth Media. Serial dilutions and subsequent plaque assays were performed. After 72 hours, wells were fixed and stained with crystal violet. Control samples with no Nanotrap particles were processed in parallel.

## Section 3: Nanotrap particles can enrich virus in clinically relevant matrices.

There are several specimens that can be used for Influenza diagnostics. These include nasopharyngeal swabs, nasopharyngeal aspirates and washes, deep nasal swabs, throat swabs, and saliva swabs and aspirates[58,87]. Our goal was to demonstrate that Nanotrap particles can enrich virus in clinically relevant matrices such as nasal swabs, nasal

aspirates and saliva aspirates. We first utilized NT120 and NT156 in a nasal aspirate scenario. We spiked Influenza A or Influenza B in 10% saliva in PBS. We sonicated the samples to break up the nasal fluid due to the viscous nature and spun samples to pellet the debris. We next incubated our two Nanotrap particles with 1ml of our spiked aspirate. Our results showed a 4 fold and 5-fold increase in Influenza A detection for NT120 and NT156, respectively (Figure 5A). We saw enrichment in Influenza B samples, albeit to a lesser extent. Incubation of Influenza B samples led to a 3.3-fold and 2.4-fold increase in viral detection with NT120 and NT156, respectively (Figure 5B). From these findings, we concluded that both NT120 and NT156 could be used to concentrate the virus in a large volume size and enhance detection of Influenza.

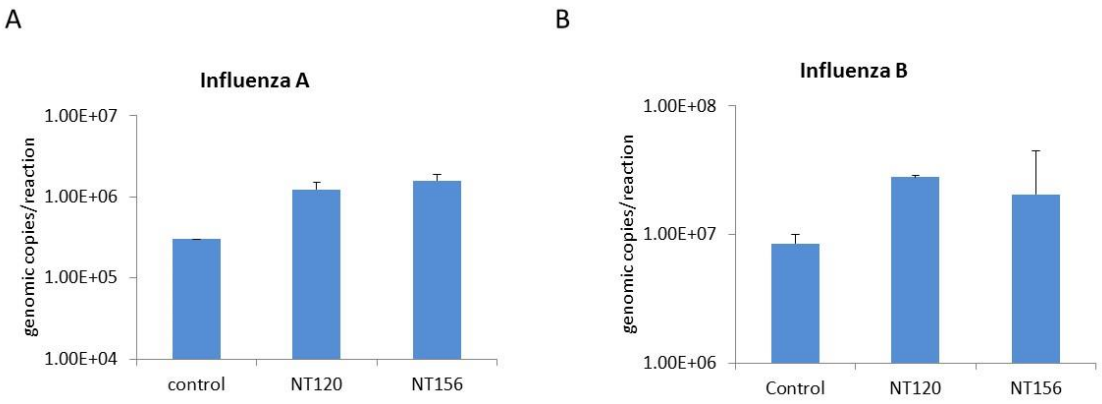


**Figure 5: Nanotrap particles can enrich virus in a nasal aspirate scenario.** Influenza A or B at 10µl was added to 900µl of PBS spiked with 10% human nasal fluid. The spiked samples were sonicated and centrifuged to pellet the debris. NT120 or NT156 were incubated with the samples for 30 minutes. Samples were then spun and unbound supernatant was removed. For panels A and B, Influenza A (A) and Influenza B (B) samples were resuspended in 50µl PBS and 130µl Ambion's MaxMax viral RNA lysis/binding solution followed by RNA extraction with the Ambion's MaxMax viral RNA extraction kit.

QRT-PCR assays using Influenza A-specific or Influenza B-specific primers and probes were subsequently performed. Control samples with no Nanotrap particles were processed in parallel.

We next coupled a mock-swab scenario with the Nanotrap particles. We spiked Influenza A or Influenza B in 10% nasal fluid and added 50µl of the spiked samples onto a swab. We then suspended the swab in 900µl transport buffer and spun down the swab head to remove all fluids. We next added the Nanotrap particles to the sample. Both NT120 and NT156 were able to concentrate both Influenza A and Influenza B. For Influenza A, we obtained a near 11-fold and 2.4 fold increase in detection for NT120 and NT156 samples, respectively (Figure 6A). For Influenza B we obtained a 6-fold and 4-fold increase for NT120 and NT156 samples, respectively (Figure 6B). Our results demonstrate that the Nanotrap particles, especially NT120, can be utilized to dramatically concentrate and increase viral detection in nasal swab scenarios.

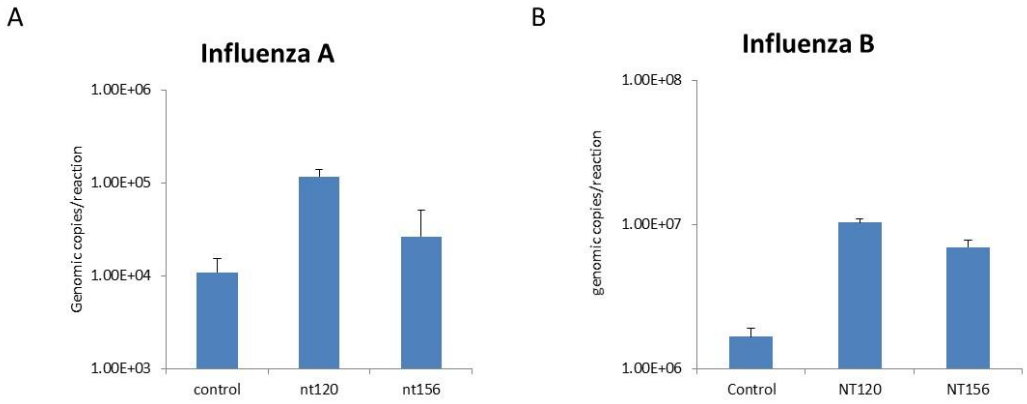


**Figure 6: Nanotrap particles can enrich virus in a swab scenario.** Influenza A or B at 10µl was added to 40µl of PBS. The Influenza-spiked PBS (at 50uL) was then spiked onto a swab followed by resuspension in 900µl transport buffer. Swab heads were spun into the transport buffer, cut into spin baskets placed above microcentrifuge tubes for maximum supernatant collection and subsequently removed. NT120 or NT156 were incubated with the samples for 30 minutes. Samples were then spun and unbound supernatant was removed. For panels A and B, Influenza A (A) and Influenza B (B) samples were resuspended in 50µl PBS and 130µl Ambion's MaxMax viral RNA lysis/binding solution followed by RNA extraction with the Ambion's MaxMax viral RNA extraction kit. QRT-PCR assays using Influenza A-specific or Influenza B-specific primers and probes were subsequently performed. Control samples with no Nanotrap particles were processed in parallel.

## Section 4: Nanotrap particles detect Influenza in the presence of other pathogens.

Clinicians oftentimes do not test for the possibility of infection with multiple respiratory pathogens. Coinfection of Influenza with other respiratory pathogens such as respiratory syncytial virus (RSV) and Coronavirus can occur. One common example is the coinfection of Influenza and RSV in children. Nanotrap particles can be utilized in the development of multiplex PCR methods in the diagnostics of respiratory tract infections. This is especially important in the investigation of the rate of co-infections as well as the

correlation of the severity of symptoms and disease[88]. We first wanted to investigate the enrichment capabilities of our acrylic acid particles in a coinfection scenario with Influenza A and B. Although rare, coinfection with both Influenza A and B types have been seen in patients[89]. Our results indicate that NT120 and NT156 can capture and concentrate both Influenza A and B when both viruses are present in one sample (Figure 7 A and B). However, detection with the Nanotrap particles in this coinfection scenario was significantly lower than what we had previously seen when capture was performed with only one Influenza type present. While NT156 enrichment averaged about 4-fold, NT120 enrichment decreased to roughly 2-fold for both Influenza A and B. These results suggest that the Influenza A and B viral particles may be competing in binding to the Nanotrap particles and perhaps saturating the Nanotrap particles that are available. Therefore, the volume of particles may need to be increased to optimize enrichment of the Influenza viruses.

We next looked at the enrichment capabilities of the acrylic acid particles with other viral pathogens. We had previously demonstrated that Nanotrap particles capture other respiratory pathogens such as Coronavirus, another RNA virus, and Adenovirus, a DNA virus[83]. We wanted to utilize NT120 and NT156 in a coinfection scenario where both Influenza A and Coronavirus are present in one sample. Our results showed a 2-fold increase in detection compared to the no NT controls (Figure 7C). Once again, these results suggest that the two viruses could be competing in Nanotrap particle binding. Secondary bacterial infections with *Streptococcus pneumoniae* oftentimes arise after infection with Influenza. We next wanted to look at whether Nanotrap particles could still

enrich virus in the presence of this bacteria. Bacteria are considerably larger than viruses, and we hypothesized that they may either severely impede Nanotrap particle binding with our virus or be too large to bind to the Nanotrap particles. Our results demonstrated that there was a 5.4-fold increase in detection compared to the no NT controls (Figure 7D), suggesting that the bacteria may be too large to bind to the Nanotrap particles. Collectively, these coinfection experiments suggest that while the Nanotrap particles will still bind to and enrich the Influenza virus, the concentration effect may be severely impeded depending on pathogen size.

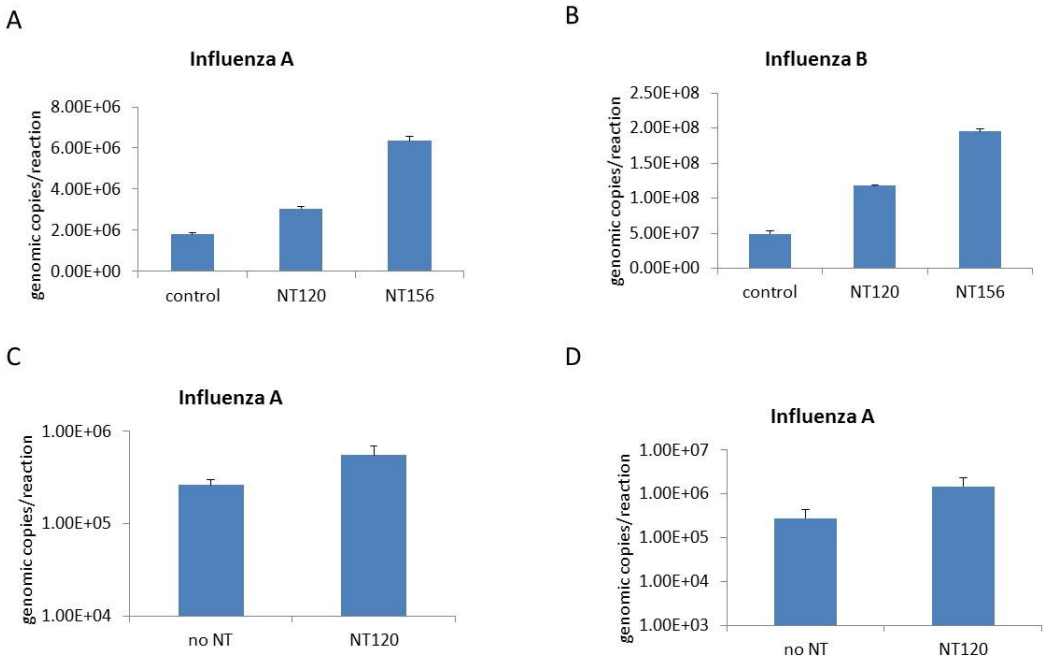


**Figure 7: Nanotrap particles detect Influenza in a coinfection scenario.** For panels A and B, PBS (at 900µl ) was spiked with 50µl Influenza A/California/2009 (to 1E+04 pfu/ml) and Influenza B/Taiwan/62 (to 5+03 pfu/ml). The spiked sample was added to either NT120 or NT156 for 30 minutes. Samples were

then spun and unbound supernatant was removed. Samples were resuspended in 50µl PBS and 130µl Ambion's MaxMax viral RNA lysis/binding solution followed by RNA extraction with the Ambion's MaxMax viral RNA extraction kit. QRT-PCR assays using Influenza A-specific (A) or Influenza B-specific (B) primers and probes were subsequently performed. Control samples with no Nanotrap particles were processed in parallel. For panels C and D, PBS (at 980ul) was spiked with 20µl Influenza A/California/2009. The 1ml sample was then spiked with human Coronavirus (C) or *Streptococcus pneumonia* SPEC1 strain (D) at a 1:2500 dilution. The spiked samples were added at a 1ml volume to 100µl NT120 and incubated for 30 minutes. RNA was extracted and analyzed by qRT-PCR as described in Figure 7. Samples containing no NT as well as samples spiked with only Influenza A (both with and without NT120) were processed in parallel.

**Section 5: Nanotrap particles can capture and stabilize Influenza viruses at ambient temperatures.**

While storage of the specimens in viral transport media helps to stabilize the virus, specimens should be shipped as soon as possible at 4$^{o}$C and are recommended to be stored at -70$^{o}$C if samples are not processed within 3 to 4 days after collection[87]. Our lab had previously demonstrated that Nanotrap particles stabilize RVFV virus at both ambient and elevated temperatures for up to 72 hours[82]. We wanted to extend these findings to Influenza. In order to determine viral stability at an ambient temperature, we incubated Influenza A and Influenza B with NT120 and NT156 at various timepoints (from 30 minutes up to 96 hours) at 22$^{o}$C. For both Influenza A and Influenza B room samples, NT120 and NT156 captured at similar viral titers over the period of 96 hours. At a steady ambient temperature, viral titers of the Influenza A control (no NT) decreased 5 to 10-fold from 30 minutes to 96-hours from a starting titer of 7E+04 pfu/ml down to 1.25E+02 pfu/ml. In contrast, both NT120 and NT156 decreased only 3 to 5-fold from

around 4E+05 pfu/ml to 7.75E+03 and 5.5E+03 pfu/ml for NT120 and NT156, respectively (Figure 8A). For Influenza B, the viral titers of the control sample slowly dropped from 30 minutes to 48 hours before dropping over ten-fold by 72 hours (Figure 8B). For NT120 and NT156 samples, there was a slight decrease in viral titer from 30 minutes to 48 hours. Furthermore, the NT120 and NT156 samples are a log higher than the control samples by 72 hours. However, while there is a still an enrichment of virus, there is a drop with the NT samples at 96 hours. These results demonstrate that NT120 and NT156 not only capture, but also stabilize the viruses at ambient temperatures for an extended period of time.

We next tested protection of the virus incubated in viral transport buffer for 96 hours at an ambient temperature. Viral transport buffers are used for the collection and transport of clinical specimens. The buffers allow the virus to remain stable at room temperature for several hours with the addition of protein (such as BSA) and sucrose for stabilization and cryoprotection, antibiotics to prevent bacterial and fungal growth, and a buffer such as HEPES to maintain a neutral pH. The BD Universal Viral Transport system was able to maintain the viability of Influenza for up to 48 hours at room temperature. We hypothesized that the coupling of viral storage in transport buffer in the presence of Nanotrap particles would further protect the virus from degradation. After 96 hours, there was a log drop in viral detection for Influenza A and a two-log drop with Influenza B without Nanotrap particles (Figure 8C). Compared to the no Nanotrap control samples, there was a 3-fold and 6-fold increase in Influenza A viral detection with NT120 and

NT156, respectively. Similarly, there was a 5-fold and 8-fold increase in viral detection for Influenza B with NT120 and NT156 (Figure 8D). These results suggest that while we are still getting an enrichment of virus with NT120 and NT156, the transport buffer contains components that protect the virus from degradation, both with and without Nanotrap particles.

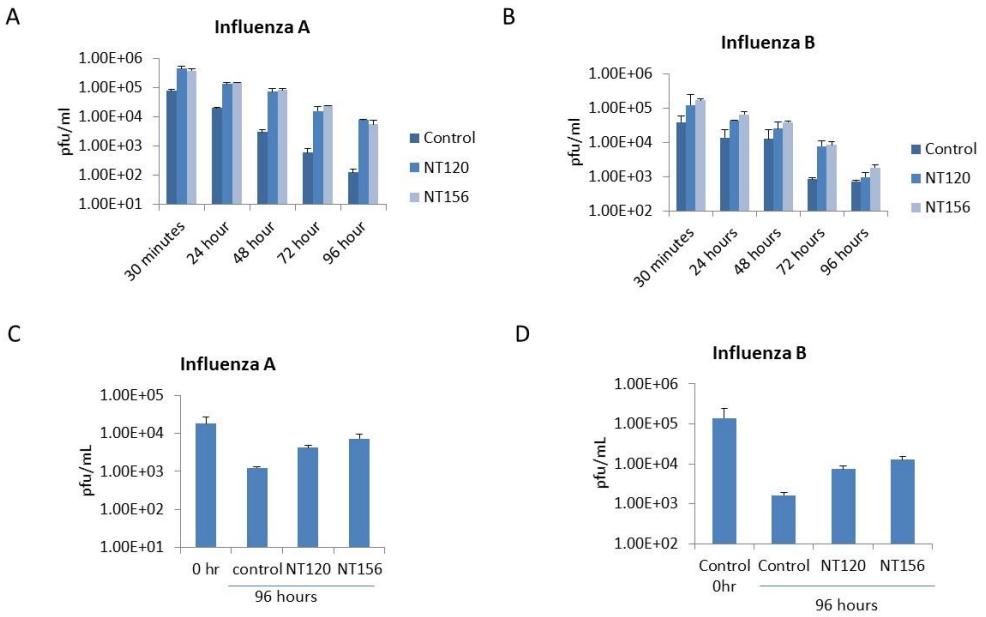


**Figure 8: Nanotrap particles stabilize virus over time at ambient temperatures.** For panels A and B, NT120 and NT156 were incubated with Influenza A (at 8E+04 pfu/ml) or Influenza B (at 4E+04 pfu/ml) in influenza growth media at room temperature from 30 minutes to 96 hours. Samples without Nanotrap particles were incubated in parallel. Following incubation, samples were centrifuged and resuspended in influenza growth media and added to MDCK cells for plaque assay analysis. For panels C and D, NT120 and NT156 were incubated with Influenza A (at 1E+04 pfu/ml) or Influenza B (at 1E+05 pfu/ml) in viral transport buffer (BD Universal) at room temperature for 96 hours. Samples without Nanotrap particles were incubated in parallel. Following incubation, samples were centrifuged and resuspended in influenza growth media and added to MDCK cells for plaque assay analysis.

**Section 6: Nanotrap particles can capture and stabilize Influenza viruses at elevated temperatures.**

In order to determine viral stability of Influenza A and B at elevated temperature, we incubated the two Influenza strains with NT120 and NT156 for 24 hours and 48 hours at a constant temperature of 37$^{o}$C. After 24-hours, the viral titers of the Influenza A control (no NT) sample decreased dramatically from 5E+04 pfu/ml down to 4E+01 pfu/ml (Figure 9A). In contrast, viral titers of samples incubated with NT120 and NT156 were significantly higher at 1E+04 and 3E+04 pfu/ml, respectively. While viral titer of the NT120 and NT156 samples drop down to 2E+02 pfu/ml at 48 hours, the viral titers remain over a log higher than the control samples, once again demonstrating a stabilization effect of the Nanotrap particles on the virus. It is important to note that while both NT120 and NT156 similarly captured and stabilized virus at room temperature, there was a difference in Influenza A capture at an elevated temperature; NT120 captured and subsequently stabilized virus at more than a log higher than NT156 samples after 24 hours incubation. After 30 minutes, both NT120 and NT156 captured and enriched Influenza B at 3.2-fold and 6.9-fold, respectively (Figure 9B). After 24 hours, control sample viral titers dropped 8-fold whereas NT120 and NT156 viral titers both dropped roughly 5-fold. By 48-hours, control sample viral titers were undetectable whereas NT120 and NT156 were detectable at 1.25E+01 pfu/ml and 6.25E+01 pfu/ml, respectively.

While viability studies with BD universal transport system were performed for Influenza at room temperature settings, there were no studies performed at elevated temperatures. Our testing with stability in transport buffer coupled to Nanotrap particle incubation produced dramatic results. The stabilizing effect of the transport buffer was considerably reduced at an elevated temperature. However, NT120 and NT156 incubation provided a 14.5- and 7-fold increase in detection, respectively, for Influenza A (Figure 9C). Likewise for Influenza B, we saw a 22-fold and 30-fold increase in detection with NT120 and NT156 incubation, respectively (Figure 9D). These stability results collectively demonstrate that NT120 and NT156 are good candidates for viral capture, enrichment, and protection of Influenza A and B, respectively, at elevated temperatures.

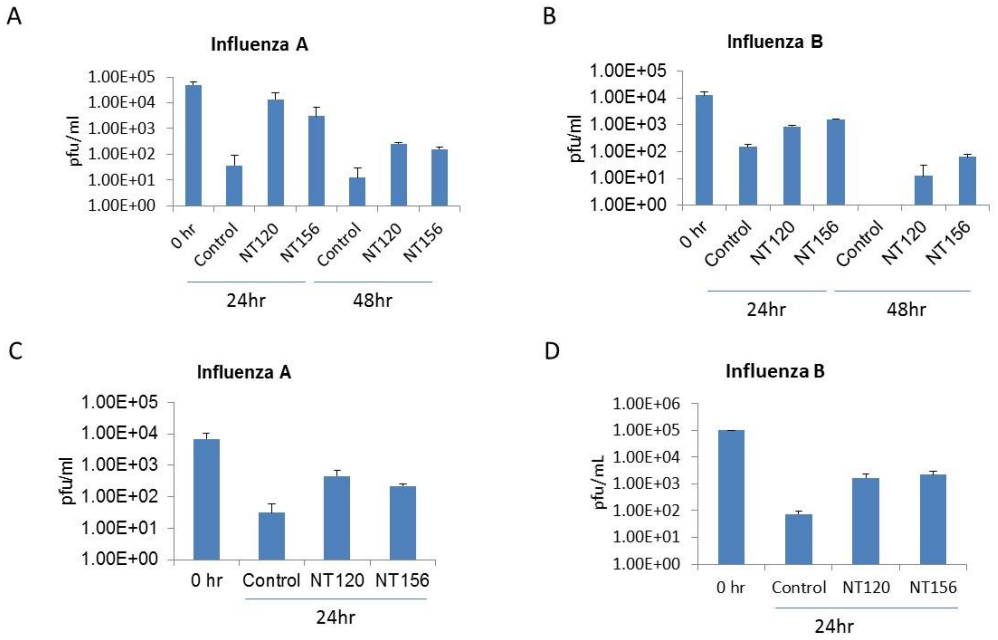


**Figure 9: Nanotrap particles preserve viral infectivity.** NT120 and NT156 were incubated with Influenza A (at 5E+04 pfu/ml) or Influenza B (at 1E+04 pfu/ml) in DMEM at 37ºC from 30 minutes to 48 hours. Samples without Nanotrap particles were incubated in parallel. Following incubation, samples were centrifuged and resuspended in influenza growth media and added to MDCK cells for plaque assay analysis. NT120 and NT156 were incubated with Influenza A (at 5E+04 pfu/ml) or Influenza B (at 1E+04 pfu/ml) in viral transport buffer (BD Universal) at 37ºC for 24 hours. Samples without Nanotrap particles were incubated in parallel. Following incubation, samples were centrifuged and resuspended in influenza growth media and added to MDCK cells for plaque assay analysis.

## Section 7: Nanotrap particles can capture and enhance detection of viral NP.

The viral NP of Influenza is commonly used in diagnostics. For example, Rapid Influenza Diagnostic Tests (RIDTs) detect the NP antigen and are commonly used by clinicians. However, the rate of false-negatives is quite high compared to molecular testing. In the next section we aimed to couple Nanotrap particle capture of virions and

viral antigens to western blotting methodology and RIDTs. We first screened five Nanotrap particles (Table 1), each with a different bait chemistry, with a 100µl volume of NP at a concentration of 8.5 ug/ml. All five particles concentrated NP (Figure 10A). We next tested the capture efficiency of the Nanotrap particles at a lower NP concentration (0.85 ug/ml) but greater sample volume of 1ml. NP is virtually undetectable at this concentration without the addition of Nanotrap particles. Again all the Nanotrap particles enriched NP, but NT45 and NT53 provided the greatest fold enrichment (69- and 180-fold, respectively) and were therefore determined to be the top two Nanotrap particle candidates for NP enrichment.

We next confirmed our findings with viral supernatants. Here it would be expected that the Nanotrap particles will be capturing virions containing NP as well as the NP from lysed virions and/or lysed cells. We incubated a 1ml volume of Influenza A/Brisbane/10 strain with our five Nanotrap particles. Two distinct bands were seen for Influenza NP. This is likely due to the ubiquitinated (top) and unubiquitinated (bottom) forms of Influenza[90]. At this viral titer, NP is was undetectable without Nanotrap particles. NT45, NT46, and NT53 samples provided up to a 15-fold enrichment of NP from viral supernatants (Figure 10C). We further confirmed our findings using cell lysates of MDCK cells infected with the Influenza A/Brisbane/10 strain (Figure 10D). We incubated infected cell lysates, which contain numerous viral and host proteins, with NT45 and NT53. Both particles provided a 3-fold enrichment compared to the no NT controls. We therefore utilized both NT45 and NT53 for future protein experiments.

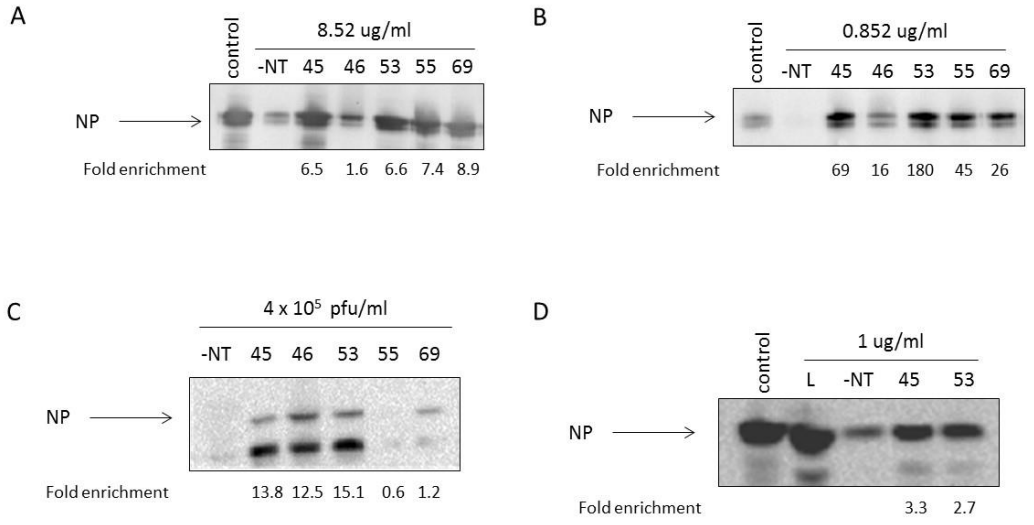


**Figure 10: Nanotrap particles capture and enrich viral NP.** A) Five Nanotrap particles (75µl volume) were incubated with 100µl of His-NP at a concentration of 8.52 ug/ml. After 30 minutes samples were centrifuged and unbound supernatant was removed. The pellet was resuspended in blue lysis buffer and boiled for 10 minutes. After 10 minutes, the sample was centrifuged and the unbound supernatant was added to an SDS PAGE gel followed by an overnight transfer. The cells were blocked in blocking buffer (3% BSA diluted in PBS and 0.01% Tween-20) for one hour. Influenza A NP antibody was diluted 1:1000 in blocking buffer and incubated with the membrane overnight at 4ºC. The next day, the membrane was washed and secondary mouse antibody was used at a 1:1000 dilution in blocking buffer. Control is no NT (10ul) at 85.2 ug/ml. B) Five Nanotrap particles were incubated with 1ml of NP at a concentration of 0.852 ug/ml and processed as described in part A. Control is no NT (10ul) at 8.52 ug/ml. C) Five Nanotrap particles were incubated with 1ml viral supernatant from Influenza A Brisbane/10/2007 (H3N2) strain at 4E+05 pfu/ml and processed as described in part A. Control is no NT (10ul) of viral supernatant. D) Cell lysates were diluted in PBS to 1 ug/ul. NT45 and NT53 (75µl volumes) were incubated with 100µl of the diluted cell lysate and processed as described in part A.

We next wanted to determine the NP limit of detection with our Nanotrap particles with both purified NP as well as in viral supernatants. We first performed serial dilutions of the viral NP at three concentrations. While –NT samples are virtually undetectable at 852

ng/ml, we see a strong band for both NT45 and NT53 samples down to 8.52 ng/ml, demonstrating a near 100-fold enrichment of our analyte (Figure 11A). We then performed serial dilutions of our viral supernatant from 1E+06 pfu/ml down to 1E+03 pfu/ml. We incubated 100µl of NT45 with 1ml of our viral supernatants at each concentration. As previously mentioned, there are two bands that are detected for NP in virally infected supernatants. Only the bottom band for NP is detectable at 1E+06 pfu/ml for the no NT samples. For the NT45 samples, there is a faint top NP band detected down to 1E+05 pfu/ml and a bottom NP band that is detected down to 1E+04 pfu/ml (Figure 11B). These results collectively demonstrate that NT45 (as well as NT53) can detect NP in both purified NP samples as well as virally infected supernatants.

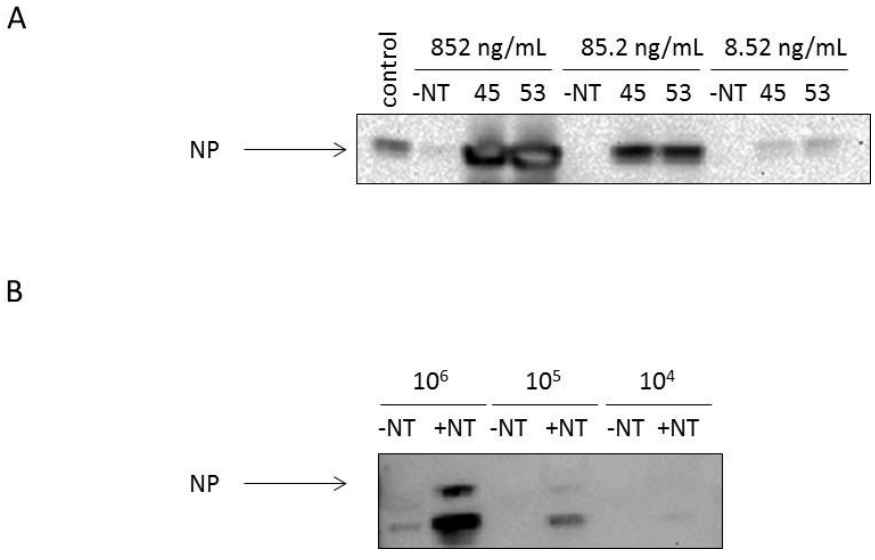


**Figure 11: Nanotrap particles enhance detection of viral NP.** A) NT45 and NT53 (100µl volume) were incubated with 1ml of His-NP diluted at 852 ng/ml, 85.2 ng/ml, and 8.52 ng/ml. Samples were processed as

described in Figure 12. No NT (-NT) samples are at 10µl volumes. Control is 8.52 ug/ml at 10µl volume. B) NT45 (100µl volume) was incubated was incubated with 1ml of viral supernatant from Influenza A Brisbane/10/2007 (H3N2) strain diluted to 1E+06 pfu/ml, 1E+05 pfu/ml, and 1E+04 pfu/ml and processed as described in Figure 10. Control samples are no NT (-NT) of viral supernatants at 10µl volumes.

**Section 8: Nanotrap particles enhance detection of viral NP in clinically relevant matrices.**

We next wanted to extend our findings to a clinical setting utilizing both nasal fluid and saliva samples. We utilized a mock nasal aspirate scenario by diluting human nasal fluid in PBS to 10% and 33%. We then spiked our viral supernatant to a final concentration of 4E+04 pfu/ml and added a 1ml volume to 100µl of NT45. Our results demonstrate that NT45 allows for the enrichment and enhanced detection of Influenza A in nasal fluid (Figure 12A). However the viscous nature of the nasal fluid mixes with the NT45 pellet and interferes with elution. Future experiments will utilize sonication to break up the nasal fluid components. We then performed a similar experiment with saliva. As previously stated, saliva is a great tool for detection as it is less invasive and easier to collect from patients. Furthermore, the components of saliva are less viscous and easier to couple with Nanotrap particles. We diluted human saliva to 10% and 33%, spiked them with virus, and incubated 1ml of the spiked saliva with NT45. While Influenza detection is slightly decreased with saliva compared to PBS alone samples, we see a nice enrichment effect of both top and bottom NP bands at both 10% and 33% saliva samples with the addition of NT45 (Figure 12B). In contrast, both NP bands are virtually undetectable without the addition of NT45 (-NT samples). These human nasal fluid and

saliva results collectively demonstrate that NT45 is a great tool to use in the collection of nasal fluid aspirates and saliva aspirates.

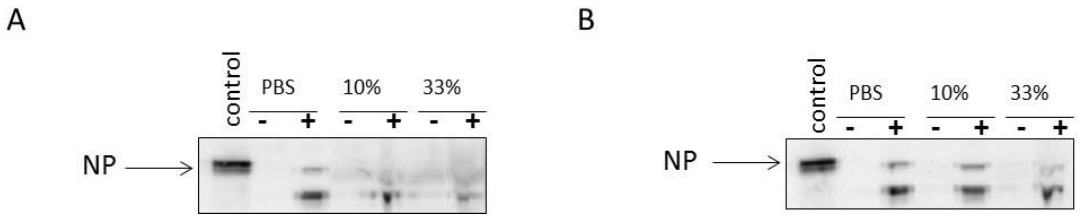


**Figure 12: Nanotrap particles enhance detection of viral NP in clinically relevant matrices.** Nasal fluid and saliva were diluted down to 10% or 33% in PBS. Influenza A/Brisbane/10 (4E+06 pfu/ml) was diluted in PBS, nasal fluid, or saliva for a final concentration of 4E+05 pfu/ml. NT45 (100µl volume) was incubated with 1ml of diluted supernatant and processed as described in Figure 10. Control samples are no NT (-NT) of viral supernatants at 10µl volumes.

## Section 9: Nanotrap particles stabilize viral NP.

As mentioned in Section 6, an important issue with sample collection is rapid degradation without the use of a cold-chain method. We performed similar experiments as Sections 6 and 7 with Influenza A/Brisbane/10. We incubated either His-NP or virally infected supernatants (both diluted in PBS) with NT45 from 48 hours to 96 hours at both ambient and elevated temperatures. The 30-minute incubations were performed at an ambient temperature. Our results show that the NP is stabilized at both ambient and elevated temperatures for up to 72 hours with the addition of NT45. At 96 hours, we start to see a

decrease in NP band intensity a both ambient and elevated temperatures in NT45 samples. In contrast, the no NT samples are virtually undetectable when samples were incubated for 30 minutes and onward (Figure 13A and B). Likewise we see a stabilizing effect with viral supernatants with the addition of NT45. Again, the no NT samples are undetectable starting at 30 minutes after incubation (Figure 13C and D). There is a change in top and bottom band detections in the ambient and elevated temperatures. For the NT45 samples incubated at ambient temperature, there is a faint top band and a strong bottom band detected from 30 minutes to 96 hours after incubation (Figure 13C). In contrast, for the NT45 samples, there is shift in band intensity from the bottom band to the top band. The top band becomes visible from 48 hours to 96 hours (Figure 13D). We hypothesize that this may be due to unubiquitinating enzymes becoming active at this temperature[90].

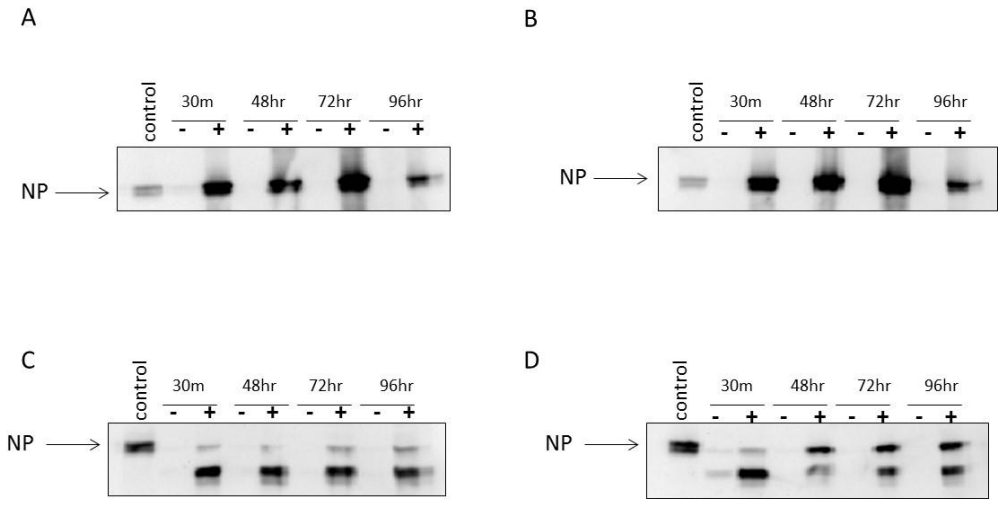


**Figure 13: Nanotrap particles stabilize virus over time at ambient and elevated temperatures.** NT45 was incubated with 1ml of His-NP at a concentration of 0.852 ug/ml from 30 minutes to 96 hours at an ambient temperature (A) or at 37ºC (B), and processed as described in Figure 12. No NT (-NT) samples at 10µl volumes were processed in parallel. Control is no NT (10ul) at 8.52ug/ml. NT45 was incubated with 1ml of Influenza A/Brisbane/10 (diluted to 4E+05 pfu/ml) from 30 minutes to 96 hours at an ambient temperature (C) or at 37ºC (D), and processed as described in Figure 10. No NT (-NT) samples at 10µl volumes were processed in parallel. Control is NT (10ul) at 8.52ug/ml.

## Section 10: Nanotrap particles can be coupled to rapid influenza diagnostic tests.

Our results demonstrated that NP could be drastically concentrated in viral supernatants. Our last goal was to couple the enrichment of NP with NT45 to the BinaxNOW® RIDT kit. Our first step was to determine if we could efficiently elute NP from the beads. The first Nanotrap particle elution buffer tested was "clear lysis buffer", which is used to extract total proteins from cells by utilizing 0.5% NP-40 detergent. We first spiked the virus at a high concentration (at 4E+05 pfu/ml) in the BinaxNOW® elution buffer to lyse the virus. We next incubated the virus with NT45 for 30 minutes then centrifuged the

sample and removed the unbound material. We resuspended the pellet in clear lysis buffer, kept the sample on ice for 30 minutes with vortexing every 5-10 minutes, then centrifuged the sample and loaded it onto the kit. We compared the results to our no NT sample that contained 120µl of the virus in BinazNOW® elution buffer. As seen in Figure 14A and B, the band indicating "Flu A" was of similar band density for both NT45 and no NT samples. We then tested detection of Influenza at 4E+04 pfu/ml with and without the Nanotrap particles. We again resuspended the pellet in clear lysis buffer and kept the sample on ice. Unfortunately, band density for NT45 sample was significantly decreased than for the no NT sample (Figure 14C and D). We therefore tested two other potential elution buffers - tissue protein extraction reagent (T-PER) and imidazole- both individually and in combination with one another. T-PER is commonly used for total protein extraction in tissue sample by utilizing a proprietary detergent[91]. We also tested imidazole, which has been previously used in the purification and elution of proteins[92]. The combination of clear lysis buffer and T-PER provided a stronger band density compared to no NT at the same viral concentration (Figure 14G). These results collectively indicated that the combination of clear lysis buffer and T-PER reagent can be coupled to RIDTs to increase detection and reduce false-negative results for Influenza and other respiratory pathogens.

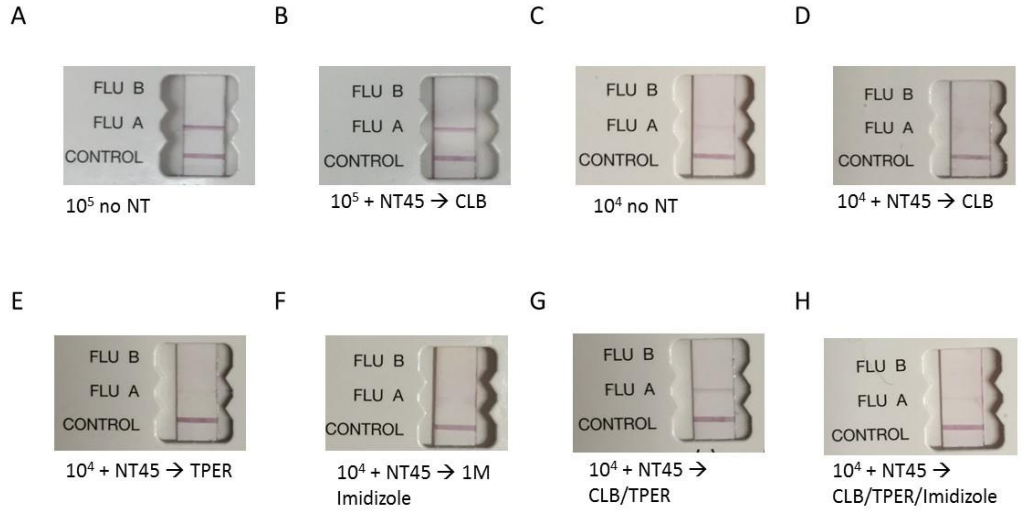


**Figure 14: Nanotrap Particles can be coupled to rapid influenza diagnostic tests.** Influenza A Brisbane/10 was diluted in BinaxNOW® elution buffer to a final concentration of 4E+05 pfu/ml (A and B) or 4E+04 pfu/ml (C through H). For no NT samples (A and C), 120µl of the diluted virus was added onto the card and read after 30 minutes. For NT45 samples, 1ml of diluted virus was incubated with 100µl NT45 for 30 minutes. Samples were centrifuged and unbound material was removed. The pellet was resuspended in 120µl of clear lysis buffer (B and D), 120µl of T-PER reagent (E), 120µl 1M imidazole (F), a 1:1 combination of clear lysis buffer and T-PER (G), or 120µl of a combination of clear lysis buffer, TPER and 1M imidazole (H). Samples were placed on ice and vortexed every five minutes for 30 minutes. Samples were then centrifuged and unbound supernatant was added onto the card and read after 30 minutes. A positive reading was indicative with a red band at the "FLU A" level.

At this point it is unclear whether the elution buffers efficiently elute NP off the beads and are just not compatible with the RIDT or if the elution buffers are not effective enough for elution. We therefore tested several of the buffers (blue lysis buffer, clear lysis buffer, T-PER, 1M imidazole, equal parts clear lysis buffer and T-PER, and equal parts clear lysis buffer, imidazole, and T-PER) with western blotting. The blue lysis buffer and imidazole provided the greatest elution and enrichment of NP (Figure 15A). After elution with the buffers, the Nanotrap pellet was resuspended in blue lysis buffer

and boiled for 10 minutes to determine how much NP was eluted off the Nanotrap particles. Matching our results in Figure 15A, there was significantly less NP detected in the blue lysis buffer and imidazole samples compared to the other four elution buffers (Figure 15B).

We then tested blue lysis buffer elution with the RIDTs. We first diluted the blue lysis buffer in the BinaxNOW® elution buffer and spiked Influenza A at $10^5$ pfu/ml. As demonstrated in Figure 15C, there is a distinct band indicative of Influenza A. However, the band intensity is weaker than the band intensity seen in for Influenza spiked in BinaxNOW® elution buffer at the same concentration (Figure14A), suggesting that components in the blue lysis buffer (e.g. SDS) may be interfering with Influenza detection. We then coupled capture of Influenza at $10^4$ pfu/ml with NT45 and Nanotrap particle elution with blue lysis buffer. After NT45 incubation, the sample was spun down and the unbound supernatant was discarded. The pellet was resuspended in 30µl of blue lysis buffer and boiled for 10 minutes. The sample was then centrifuged and the unbound supernatant was brought up to a volume of 120µl in BinaxNOW® elution buffer. Unlike the faint bands detected at this viral titer with the clear lysis buffer/T-PER samples (Figure 14G), no Influenza A band was detected (Figure 15D). This suggested that the incompatible components of the blue lysis buffer as well as an unrecognizable conformational change of NP due to boiling does not allow for the protein to be detected by the RIDT methodology. Taken together, the western blot and RIDT results demonstrate that blue lysis buffer and imidazole are best for elution of NP from the

beads, but are not compatible with the RIDT. Future studies will utilize other elution buffers that provide up to 100% elution of NP off the beads while being compatible with RIDTs.

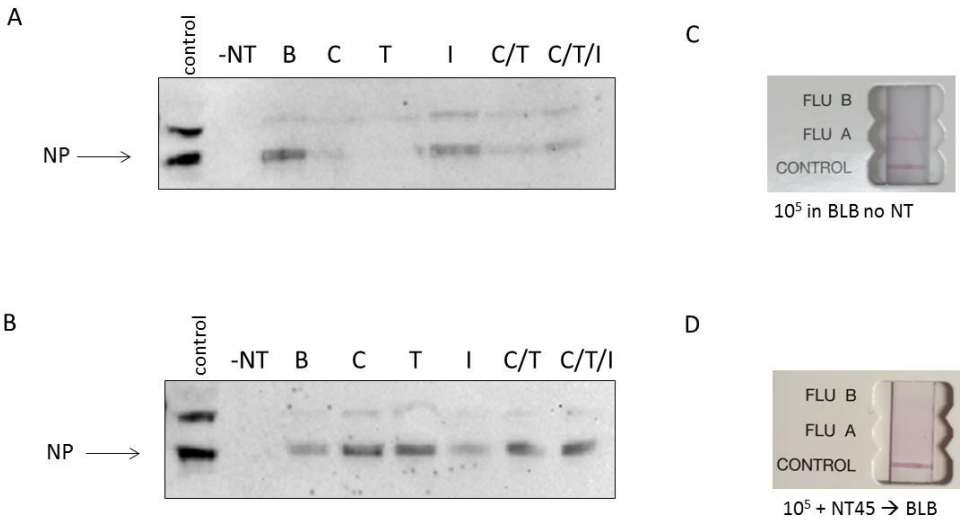


**Figure 15: Blue lysis buffer and imidazole elute NP but are not compatible with RIDTs.** In panel A, 1ml of Influenza A/Brisbane/10/2007 strain at 4E+04 pfu/ml was incubated with 100µl NT45 for 30 minutes. Samples were then centrifuged and resuspended in various buffers. The samples incubated on ice for 30 minutes with vortexing every five minutes. The samples were then centrifuged and unbound supernatant was resuspended in 6µl 4X LDS buffer and 1µl 1M DTT. Samples were heated at 72oC for 15 minutes, centrifuged and loaded onto an SDS PAGE. In panel B, the eluted pellets from panel A were resuspended in 30µl blue lysis buffer and boiled for 10 minutes. The samples were centrifuged and the unbound supernatant was loaded onto an SDS PAGE. Control is at 4E+05 pfu/ml (10µl volume). Abbreviations are as follows, -NT is no Nanotrap particle at 10µl volume, B is blue lysis buffer, C is clear lysis buffer, T is T-PER reagent, I is 1M imidazole, C/T is equal parts clear lysis buffer and T-PER, and C/T/I is equal parts clear lysis buffer, T-PER, and 1M imidazole. The samples were processed via western blotting as described in Figure 10A. For panel C (no NT sample), 20µl of virus was diluted in 45µl of blue lysis buffer and 135µl elution buffer from BinaxNOW® kit. A volume of 120µl was then added onto the card and read after 30 minutes. For panel D (NT45 samples), 1ml of the diluted virus was incubated with 100µl NT45 for 30 minutes. Samples were centrifuged and unbound material was removed. The pellet was resuspended in 30µl of blue lysis buffer (D). The samples were boiled for ten minutes. Samples were then centrifuged and unbound supernatant was brought up to a volume of 120µl with BinaxNOW® elution buffer, added onto the card, and read after 30 minutes. A positive reading was indicative with a red band at the "FLU A" level.

# CHAPTER EIGHT: DISCUSSION

Nanotrap particles have traditionally been used for the capture and enrichment of proteins [3,4,80,93,94]. Several papers have shown that these hydrogel particles enrich numerous proteins such as insulin, myoglobin, and PDGF [3,80]. A 2011 paper by Douglas et al demonstrated that the Nanotrap particles can dramatically concentrate Lyme disease antigens in urine and enhance detection at previously undetectable concentrations [94]. The Nanotrap particles can also be used for the capture of virions. In 2013, we demonstrated the capture and enrichment of virions from RVFV and other viral pathogens with several Nanotrap particles [82]. Importantly, we showed that while the virus can be inactivated with heat or detergent after Nanotrap particle incubation, the nucleic acid of the virus is still detectable with qRT-PCR methodology. We have expanded upon these previous findings and shown here that the Nanotrap particles can be utilized as a sample preparation tool to concentrate Influenza. Specifically, infectious virus can be concentrated from a large starting volume of sample into a small volume from clinically relevant matrices, as demonstrated by plaque assay, qRT-
PCR, and western blotting.

Our first goal was to determine the compatibility of the Nanotrap particles with the Influenza virus. From our initial Nanotrap particle screenings we identified NT55 as our top candidate (Figure 1A and B). NT55 is an acrylic particle that is roughly 800 nm in diameter. We further tested other acrylic acid particles of various sizes and

conformations and discovered that NT120, which consists of 10% methylacrylate saponified to acrylic acid, performed the best. NT120 is smaller than NT55, at roughly 590 nm in diameter. We have hypothesized that the Nanotrap particles bind to the virus via the virus' surface glycoproteins. The acrylic acid bait of the particles attracts polypeptides and proteins that have a net negative charge[93]. Interestingly, the host cell membrane and sialic acid receptors have a net negative charge while HA1 subunit of human Influenza A typically has a positive charge[95]. Alternately, the Nanotrap particles could be binding to the negatively charged zone in the neuraminidase active site[96]. However, the exact mechanism by which the nanoparticles bind to the Influenza virus is still unknown. We tested the enrichment capability of NT120 in three other types of Influenza A subtypes and strains. In contrast to the Influenza A/California strain, we observed less enrichment of the other three Influenza A viruses with NT120 capture. This suggested that, due to the variations in the HA and NA, other Nanotrap particles might perform better at enriching other subtypes and strains of Influenza A.

We hypothesize that the creation of a considerably larger Nanotrap particle will provide a greater surface area for more virions to bind to. To test this hypothesis, we compared capture of NT120 with a significantly larger particle called NT156. NT156 is over 3000 nm in diameter (5X larger surface area compared to NT120) and contains a non-cross-linked interpenetrating network surface that resembles arms sticking out of the particles. Here we speculated that the “arms” may help for more viral particles to stick to one Nanotrap particle. Both particles provided 6-fold enrichment in detection of virions for

both Influenza A and Influenza B. At this point, the similarities in capture of NT120 and NT156 did not confirm our hypothesis that a larger surface area leads to more virion binding. We therefore utilized both Nanotrap particles to determine their concentration effects in clinically relevant matrices and coinfection scenarios.

A common problem in diagnostics is the interference of high abundant host proteins. Nasopharyngeal samples contain viscous analytes that non-specifically bind to antibodies and significantly decrease detection. Furthermore, nasal aspirate collection dramatically dilutes the sample. Nasopharyngeal wash samples are typically collected by inserting 2-4 ml of PBS or similar saline solution into each nostril with a syringe. The head is then tilted forward and the solution is collected in a specimen container. A total of 3-8 mls can be collected from one patient[97,98]. However, molecular assays and RIDTs allow only the testing of 50-120µl volumes of sample. Our nasal aspirate experiments utilized 1ml volumes of samples with our Nanotrap particles. We demonstrate that while we see a drop in detection compared to PBS alone, there is significant enrichment of both saliva and nasal aspirate samples with Nanotrap particles with both qRT-PCR and western blotting assays as demonstrated in Figure 5 and Figure 12, respectively. Saliva samples are less commonly used for Influenza diagnostics. However, several studies have shown that, compared to nasopharyngeal swab and aspirate samples, this type of specimen is less invasive, easier to collect and just as reliable as nasopharyngeal samples[60]. Therefore, our future studies will utilize saliva samples in the development of Nanotrap particles coupled to lateral flow assays.

Another issue in diagnostics is the degradation of samples soon after collection. This is due to several factors that include both endogenous and exogenous proteases, as well as temperature and pH changes. It is therefore recommended that samples be placed in temperatures of 4°C or lower for short-term transport and at -80°C for long-term storage [99]. However, these temperature settings are not always possible in a field environment. Nanotrap particles have previously been found to protect analytes from proteolytic degradation by trapping the protein of interest within the particles while simultaneously sequestering proteases such as trypsin [80,93]. Furthermore, the hydrogel particles shrink at elevated temperatures above 34ºC [100], further trapping the analytes within the particles. We have expanded upon these findings by demonstrating that the Nanotrap particles can not only capture and enrich both Influenza virus as well as viral NP in the presence of abundant resident proteins that are found in nasal fluid and saliva, but also protect both the virion and the viral antigen from degradation for up to 120 hours at both ambient and elevated temperatures, as demonstrated by results shown in Figure 9 and Figure 13 for virion and viral NP, respectively. This sample preparation technology will allow for sufficient collection and transfer of clinical samples from a field or hospital setting to a laboratory for subsequent testing.

Our screening of NP via western blotting identified NT45 and NT53 as our top two candidates, both of which are triazine-derived textile dyes that have commonly been used for protein purification via affinity chromatography [101]. The structure of the dyes mimic

the structure of substrates that bind to the active sites of different proteins, and are therefore able to bind a wide variety of proteins. Dye affinities are determined by the structure of the dye and how well it can bind to the active site of the proteins in question. NT45 is one core particle that is coupled to two different dyes; it is a mixture of the reactive red dye and a reactive yellow dye, whereas NT53 contains the cibacron blue bait. Cibacron blue has been described as a "universal pseudoaffinity ligand" that nonspecifically binds analytes through ionic and/or hydrophobic interactions due to its aromatic (nonpolar) and sulfonate (ionic) groups [102]. Influenza NP interacts with a variety of macromolecules. While the exact mechanism in which the Nanotrap particles bind to the virion or analyte is unknown, we hypothesize that the baits of the Nanotrap particles are somehow attracted to the NP in a similar fashion as the interactions between NP and other macromolecules[103].

Our results have demonstrated significant enhancements in terms of both sample enrichment detection and subsequent protection of Influenza virion as well as viral antigens with Nanotrap particles, when utilizing plaque assays, qRT-PCR, and western blots, and indicates that our results could easily be expand to other downstream assays such as lateral flow assays (LFA) and enzyme-linked immunoassays (ELISAs), both of which can be used to detect the presence of viral NP. Sensitivity issues are a common problem during both LFAs ELISA diagnostics as viral titers often fall below the threshold of detection. While viral titers in Influenza-infected individuals can be very high, titers start to decrease after five days in otherwise healthy adults. For lateral flow

assays, samples must be collected during a small window of time (no more than 4-5 days) or false-negative results may be rendered[47]. Our western blot results have demonstrated up to a 100-fold increase in NP detection after Nanotrap particle incubation, as demonstrated by western blotting. Specifically, our limit of detection experiments demonstrated the enhanced detection of the NP after Nanotrap-particle incubation at viral titers that are previously undetectable and would have rendered a false-negative result. We anticipate similar results with the lateral flow assays and Ag-capture ELISA methodology. Our future goals are to utilize the Nanotrap particles to exclude high-abundant molecules while concentrating the NP in high-volume samples (up to 10 ml). Our aim is to increase the sensitivity range for both Ag-capture ELISAs to be comparable or exceeding that of current PCR methodologies. As demonstrated in Figures 16-20, many buffers that are commonly used to elute samples off of the Nanotrap particle interfere with antigen-antibody capture for both LFAs and ELISAs. Therefore, our goal is to find a proper elution method that efficiently elutes our sample off of the particles and is compatible with ELISAs. Newer generations of Nanotrap particles that can be "degradable" may be the solution to coupling the particles with this assay.

While the promiscuity of the Nanotrap particles allows for more than one virus or analyte to be captured, our results demonstrated that this feature does not interfere with the Nanotrap particle's enrichment capability. This feature of the Nanotrap particles is favorable when the cause of infection is unknown as several viruses can result in the same symptoms. An example of this is with Influenza and many cold viruses such as

Adenovirus or Coronavirus. While our lab has previously shown that Nanotrap particles work in a mixed infection scenario with RVFV and HIV coinfection[82], we wanted to extend those findings to respiratory pathogens. Coinfection of Influenza A and B are rare but have occurred. It is important to distinguish between the two strains since new strains can arise from genetic reassortment. Our results show that the Nanotrap can capture and detect both Influenza A and B in one sample. We next wanted to demonstrate that the Nanotrap particle concentration effect is not hindered in a coinfection scenario with another virus (Coronavirus) or with bacteria (*Streptococcus pneumoniae*). Again, we do not see a compromise to detection of Influenza A in the presence of the bacterial pathogen. However, there is a decrease in fold-enrichment of Influenza A in the presence of Coronavirus, suggesting competition of the two viruses for Nanotrap particle binding. A potential solution to this issue is to increase the volume of Nanotrap particles to allow for more virions to bind. The Nanotrap particles' ability to concentrate various antigens and/or virion allows for analysis using various multiplex assays (e.g. multiplex PCR, Luminex) containing a large panel of primers or antibodies targeted against pathogens that induce these similar symptoms.

The Nanotrap particles also serve as an important tool for host biomarker discovery. As previously mentioned, the promiscuity of the Nanotrap particles allows for the enrichment of both host and viral antigens from a single sample. Since host protein biomarkers are typically of low abundance and size, they are easily missed in favor of large, high-abundant molecules that make up the majority of the circulating proteins, with

albumin itself accounting for 55% of the proteins found in plasma samples [104]. This is a common problem with mass spectrometry, which requires a very small sample size and a starting mass in the range of 0.1–10 µg per protein [105]. The size sieving property of the Nanotrap particles work to exclude large biomolecules while concentrating small, low-abundant biomarkers. A number of recent papers have demonstrated the enhanced detection of several biomarkers with Nanotrap particles. A 2009 paper by Longo et al demonstrated the detection of the platelet-derived growth factor protein at previously undetectable levels[106]. Another paper published by Tamburro et al demonstrated the sequestration and concentration of the host cytokines interleukin-6 (IL6) and interleukin-8 (IL8) with Nanotrap particles. Importantly, the proteins are protected from enzymatic degradation [4]. These findings can be expanded to biomarker discovery of various respiratory pathogens.

We have demonstrated that the Nanotrap particles are a reliable and quick sample preparation technology that allows for the enrichment and detection of a specific protein target from a large sample volume, the exclusion of larger molecular weight proteins, and the protection of the protein from degradation over an extended time and at elevated temperatures. Concentrated samples can then be efficiently eluted with only a small volume of eluent and processed with assays such as plaque assays or western blotting for further analysis. Our future projects will investigate the feasibility of extending this concept to other downstream methodologies, which will include lateral flow assays, ELISA, mass spectrometry, and Luminex assays. Lastly, we hope to couple the Nanotrap

particles to point-of-care diagnostic devices that can rapidly (<30 minutes) detect various respiratory pathogens. We also aim to expand this concept to numerous emerging infectious diseases and provide a quick, safe, and easy-to-use tool for diagnostics in a field and clinical setting.

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