U.S. DEPARTMENT OF ENERGY

PNNL-20571

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

# Antibody-Based Diagnostics and Production for High Consequence Animal Pathogens

## Final Report for CRADA PNNL/176 with New Horizons Diagnostic Corporation

Larry Loomis

July 2011

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# Contents



# Table of Figures

# 1.0 Background

New Horizons Diagnostic Corporation (NHD) develops and manufactures testing products for the detection of bacteria and toxins in human, environmental, surface, food, and water samples for government, municipal and corporate customers. It offers diverse medical products, such as products for testing cholera, gonorrhea, syphilis, and drug abuse screening products. The major product line for NHD is the development, marketing and sale of rapid antibody based diagnostic kits. World Health Organization (WHO) studies have shown that since 1996, infectious and parasitic diseases accounted for 43% of the 40 million deaths in developing countries. In the US alone, the death rate due to infectious diseases rose 58% from 1980 to 1992. The incidence of food-borne illness continues to rise. Emerging infectious diseases in animals with the potential to afflict human populations, such as BSE and avian influenza, continue to increase in number as well. As many as 81 million illnesses and 9,000 deaths occur each year in the US from food-borne microorganisms. In addition, pesticides in food give rise to an estimated 6,000 cancer cases annually in the US. The Environmental Protection Agency (EPA) estimated, in the early 1990s, that approximately 43% of the water systems serving 120 million people had EPA violations, and nearly one million people became ill due to contaminated water. The company also provides bio threat detection products, including diagnostic kits, collection kits, and environmental kits. The collaborative effort, if successful, will permit the identification and development of new and improved diagnostic kits for the targeted diseases. New Horizon Diagnostics matched DOE's dollars through a Cooperative Research and Development Agreement (CRADA). That CRADA is the subject of this report. The proposal was ultimately funded and the CRADA between NHD and PNNL was established. PNNL managed the activities at the Russian State Diagnostic and Prevention Center for Human and Animal Diseases (DPC) and its collaborators (Ivanovsky Virology Institute, the All-Russian Institute for Animal Health (ARRIAH), and Gamaleya Institute) to support the efforts between NHD and DPC and it collaborators in developing commercially viable diagnostics and therapeutics.

# 2.0 Progress

The goal of this project was to use existing data and archived inventories for a series of antigens and antibodies (animal disease agents and toxins) for developing new rapid diagnostic kits (3-5 min.) for animal, environmental, and food-borne disease detection and therapeutic reagents for treating livestock diseases. NHD has global distribution and sales networks for both low-end and high-end diagnostic kits and treatments. The project is now complete. One of the major results obtained during the performance of this project was the configuration of diagnostic kit in fast- track format that targeted the different Avian Influenza Virus (AIV) Type A strains, including the highly pathogenic (both for birds and human) H5N1 serotype. The AIV- Ag dip-stick format kit was assembled from monoclonal antibodies and antigen developed in both the United States (USA) and, in parallel, in the Russia Federation (Moscow, DPC). At present, the specificity and sensitivity of the device is being validated in several countries, including those in Southeast Asia and republics of the Former Soviet Union.

Another major accomplishment was development of reagents capable of detecting the non-structural proteins of Foot and Mouth Disease virus (FMDV), and having the ability to differentiate naturally infected animals from vaccinated animals. This work was performed by ARRIAH (Vladimir) and DPC (Moscow) scientists using the dip-stick format. The work was coordinated by NH which also arranged for validation and certification of disease diagnostic kits byte FDA, CDC and USDA.

During Phase I and II activities, eight (8) disease targets were selected for development of antigen/antibody diagnostic systems. NHD's plan during Phase I was to prioritize their needs (based on the state and availability of antigens/antibodies/therapeutics for specific pathogens and toxins, the need for conducting the necessary reduction-to-practice efforts, and market drivers). This prioritization revealed the importance of getting at least two of these diagnostic kits into the marketplace as quickly as possible in order to meet existing and emerging animal disease threats either due to natural outbreaks or malignant intent (agro terrorism).

Diagnostic systems developed under this CRADA:

Under the direction of DPC and ARRIAH the following reagents were developed and produced:

1. A full-length recombinant bovine prion protein (rbPrPc) and recombinant bovine prion antigens were constructed and produced using an *E. coli* expression system, verified based on sequence homology, characterized for immunogenicity and selected as appropriate antigens to produces monoclonal antibodies against recombinant protein bPrP conformers for ELISA formats based on the specificity and avidity of the anti-PrPc monoclonal antibodies (Figure1). Specific monoclonal antibodies to prion proteins (PrP) for Transmissible Spongiform Encephalopathy (TSE) diagnostic tools were developed for use in ELISA formats and/or possible lateral flow assay systems. Newly generated monoclonal antibodies produced in response to recombinant PrP protein was characterized for specificity against major TSE infectious agents ($PrP^{d}$) (bovine spongiform encephalopathies, (BSE), scrapie (sheep), and lethal human variant of Creutzfeldt-Jacob disease (nvCJD)). This evaluation process yielded several selected monoclonal clones with strong positive reactivity for the detection of infectious prion isoforms of human or animal origin. Specificity was confirmed using immunobloting studies and electron microscopy immunogold histological staining. These activities were completed with the development of an appropriate ELISA assay system for the detection of $PrP^{bse}$ antigens in real, non-fixed BSE infectious prions in brain tissue. The ultimate objective is, by optimizing conditions and improving sensitivity, to develop "pan-prion" antibody reagents that will reliably detect TSEs infectious agents ($PrP^{sc}$, $PrP^{BSE}$ and $PrP^{CJD}$) in brain samples of different species. Following completion of this task, the investigators made available PrP antigen and monoclonal antibodies to the commercial partner for verification studies.

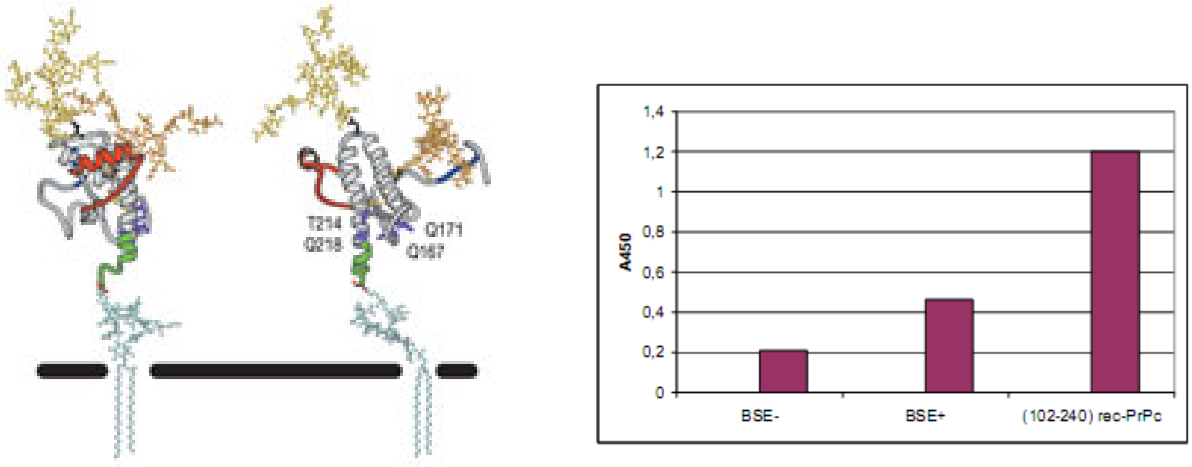
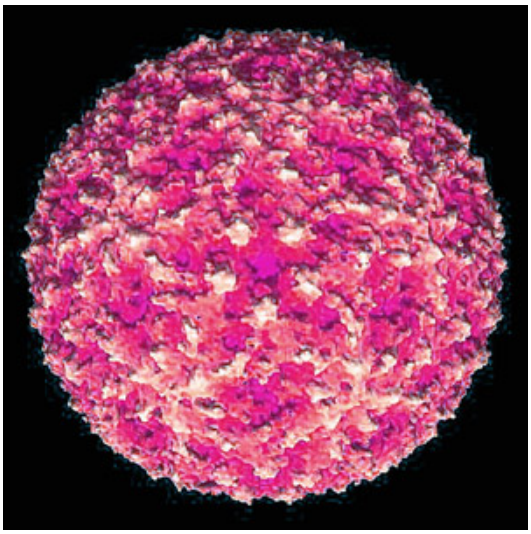
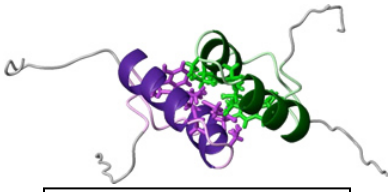



**Figure 1: Recombinant Prion Protein molecular structure and assessment of specificity of monoclonal antibody-based sandwich ELISA Assay system**

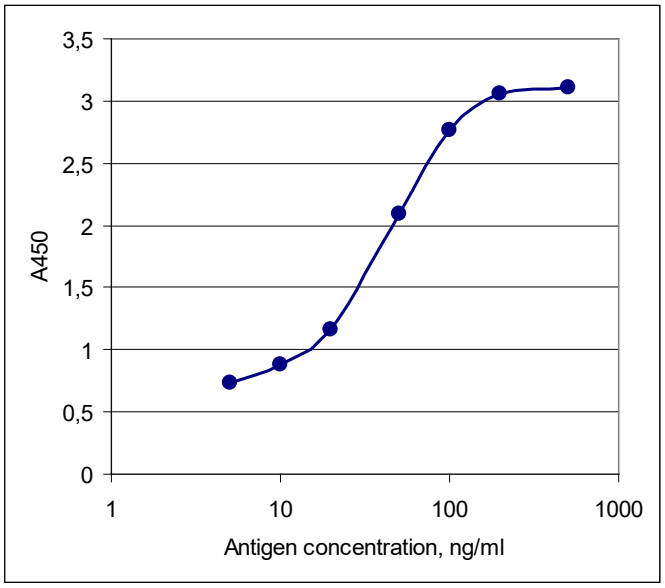
2. The next task focused on the development and production of an FMDV recombinant nonstructural protein antigen. In this study the recombinant nonstructural 3A, 3B, and 3AB proteins of FMDV were obtained, and methods and procedures identified for their synthesis and purification were developed and optimized. Using the recombinant proteins in indirect ELISA as antigens, evidence was obtained that all three proteins were suitable for differentiation of vaccinated and FMDV-infected cattle, but ELISA assays based on the observation that the 3A protein had greater specificity and sensitivity than 3B- and 3AB-ELISAs. These data supported the conclusion that FMDV 3A recombinant protein was the most promising antigen for developing a differentiating assay.



FMD Virus



3A recombinant protein




**Reactivity of recombinant 3A (rec-3A) protein in sandwich-ELISA with Mabs 1G12 and 5D11**

**Figure 2: FMD virus, 3A recombinant nucleoprotein and immune-reactivity**

3. The third task conducted by DPC and the Ivanovsky Institute, was to produce and supply Avian Influenza A recombinant nucleoprotein (rNP antigen) and anti-rNP monoclonal antibodies for specific diagnostic kits. A baculovirus expression system was employed using an appropriately designed NP gene construct that resulted in properly folded AI virus rNP antigen. Using this system, the investigators were able to synthesize and isolate sufficient material for the development of monoclonal antibodies and also supply material for evaluation by the commercial partner. Purity was established using SDS-PAGE electrophoresis with immuno-reactivity and specificity of the rNP antigen confirmed using Western blot and indirect ELISA analysis. Chicken anti-NP positive and negative sera and numerous other sera from birds infected with AI virus were analyzed. This included H5N1 serotypes. See Figure 3. For monoclonal antibody development, the focus was detection reagents (antibodies) and methods to demonstrate and characterize the effectiveness of the antibodies to bind with native and recombinant NP proteins. During this process six stable

hybridoma cell lines were identified for evaluation with at least two clones producing monoclonal antibodies interacting with both antigenic types (recombinant and native AI virus proteins). Sufficient antibodies and recombinant proteins were transferred to the commercial partner for evaluation.

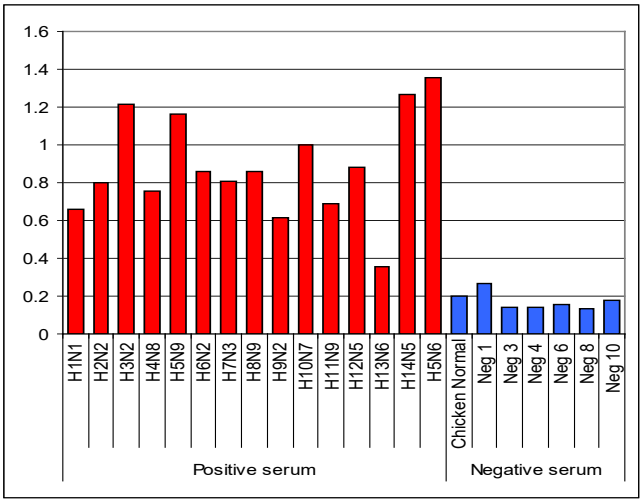



**Figure 3: Immunoreactivity and specificity of recombinant NP protein assessed against a panel of positive and negative reference chicken sera and sera samples from birds infected with H5N1 and H1N1 and other type A virus.**

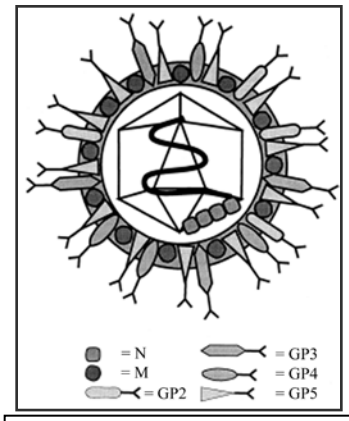
4. The fourth task dealt with the selection and recombinant production of a Clostridium perfringens specific exotoxin antigen with the most relevant application being the identification of human and animal diseases. It should be noted that it is not sufficient to just identify the presence of Cl. Perfringens. It is also important to identify the bacterial strain and toxin type and quantity since that information is essential in disease management. The toxin selected was the epsilon toxin produced by both type B and D strains of Cl. perfringens. Classical approaches were used to produce hybridoma cells secreting monoclonal antibodies to the truncated recombinant epsilon exotoxin. In this system mice are immunized with the antigen but the production of primed spleen cells were not successful. It should be noted that the use of gluteraldehyde or formaldehyde antigen treatment lead to aggregates and the masking of antigenic determinants. This issue was not resolved when the project was completed without the production of monoclonal antibodies against this exotoxin.

5. The fifth task addressed was Classical Swine Fever virus (CSFV). The research included the identification, isolation and synthesis of the recombinant E2 antigen and the production of monoclonal antibodies for incorporation in ELISA and/or lateral flow diagnostic systems. Classical swine fever is an economically important highly contagious disease mainly in swine but also causes disease in cattle and sheep. Since vaccination with live attenuated CSFV protects pigs from disease, it becomes economically important to differentiate vaccinated swine from chronically ill as both population exhibit antibodies to the disease in their blood. To address this detection goal, extensive molecular characterizations of CSFV strains, geographic isolates, vaccine strains and recombinant E2 antigen were analyzed using PCR methodologies including restriction analysis of PCR products. This also included antigenic and immunogenic characterization of the CSFV antigens, and assessment of protection of experimental swine from lethal challenge using different vaccine procedures. The recombinant CSFV structural virus glycoprotein gp55 (E2) antigen was selected as the target antigen and produced from cloned genes in vector plasmids using E. coli and baculovirus expressions systems. These prepared E2 antigens were evaluated against a panel of field experimental CSFV-positive antiserum samples and served as the challenge antigens for the development of monoclonal antibodies with specificity for the CSFV. The second element of this study was the development of anti-E2 specific monoclonal antibodies and the selection and validation of these antibody preparations against known CSFV strains, field isolates, serum and tissues from infected animals. Following antibody evaluation of 21 cloned cell lines production monoclonal antibodies, 2 stable hybridoma clones were identified for inclusion in ELISA assay system that meet the defined criteria as note previously. E2 antigen and sufficient monoclonal antibodies were provided to commercial partner for evaluation.

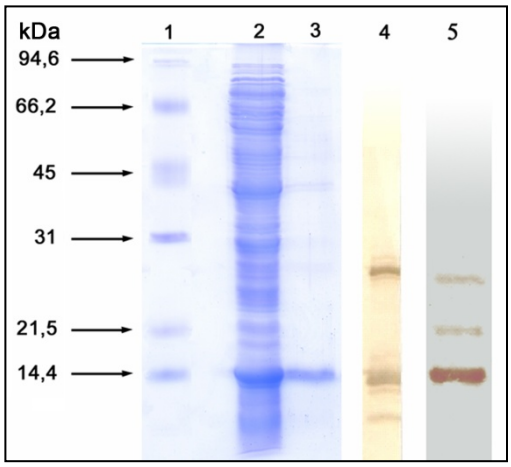
6. The sixth task called for development of diagnostics for Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). This was conducted by DPRI. As before, the focus was the development of appropriate reagents for diagnostic tools using ELISA and/or lateral flow assay systems. As had been the approach in other diagnostic systems, a marker protein was identified that would serve as a universal antigen for immunoassays detecting virus strain-specific antibodies. Based on extensive research conducted by DPRI and others on

identifying an appropriate stable marker protein for PRRSV, the choice was the nucleocapsid protein rN (ORF7 gene). This decision were based on sequence data publicly available or determined for American, Russian (Asian) and so called European subtypes of PRRSV. The processes and procedures for the preparation of the selected recombinant rN protein included the isolation of the ORF7 genes from PRRSV strains, cloning the genes and expression of gene products using either E. coli or the baculovirus expression systems. Following purification by affinity chromatography, the recombinant protein was characterized for purity using SDS PAGE electrophoresis and immunological specificity was confirmed using ELISA and Western blotting analysis with a panel of reference sera.

The production of monoclonal antibodies against PRRSV recombinant was accomplished using the rNP protein from the PRRSV European subtype I as the challenge antigen for hybridoma preparation. During this process, twelve (12) stable hybridoma clones were identified from which two clones were selected for the final assay products. This decision was based on extensive assessment of the specificity and affinity of the antibodies using Western blot analysis and the activity of the antibodies in direct, indirect and sandwich ELISA assay model systems. Following completion of this task, the required r- nucleocapsid protein and monoclonal antibodies were supplied to commercial partner for further joint investigations.



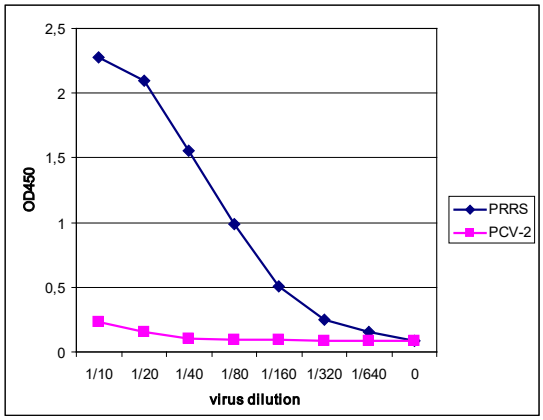

Scheme of PRRSV proteins assembled in virus particle




SDS-PAGE and Western blot analysis of recombinant nucleocapsid proteins produced in pET and (2, 3, 4) and Bac-to-Bac (5) expression systems.

| Antigen/ Virus strain | Mab's ## | | |
|---|---|---|---|
| | **E10 (NC1)** | **CS** | 2E5 |
| NADC -8 (NA, Type 2) PRRSV (USA) | | | + |
| NADC -9 (NA, Type 2) PRRSV (USA) | | | + |
| NVSL-14 (EU, Type I) PRRSV (Russ) | | | + |
| 45+Voronezh (EU, Type I) PRRSV (Russ) | | | + |
| PCV2 (Russian isolate) | | | - |
| TGEV (Russian isolate) | | | - |
| PEDV (Russian isolate) | | | - |
| CSFV (Strain CS, Russia) | | | - |
| PRV (Russian Isolate) | | | - |

Test antigens for specificity determination




Titration curves of PRRSV culture preparation in sandwich ELISA

**Figure 4: PRRSV virus, recombinant proteins characterization, specificity and immune reactivity**

7. The seventh task dealt with the development of diagnostics for *Pasteurella multocida* which was done by the Diagnostic and Prevention Institute for Human and Animal Diseases (DPRI) and the All-Russian Institute of Animal Health (ARRIAH). The developed reagents will be used for diagnostic ELISA and/ or lateral flow assay systems. The veterinary importance of this disease is its ability to cause primary and secondary infections in all domestic animals, especially cattle, swine, sheep and goats with severity

including abortion, meningitis and encephalitis. Since there are numerous species identified and being added to the genus *Pasteurella* on an annual basis, it was essential that the assay system be able to identify those subspecies capable of causing significant disease. Such is the case for *Pasteurella multocida* subspecies. After an extensive literature review and careful comparison of known sequences for possible marker proteins, the investigators selected the OmpH protein as the antigen target for developing a pan- antigen for most diseases caused by *P. multocida* strains. As with prior test systems developed during the performance of this project, the researchers determined the appropriate genetic sequence for the antigen to establish a stable recombinant protein for the standardized assay system. For these studies, *Pasteurella multocida* strains of serogroups A, B and D were supplied by ARRIAH from their culture collection. The OmpH gene was isolated, cloned into an expression plasmid and expressed in an *E. coli* system. The resultant proteins were isolated using affinity chromatography and analyzed using SDS-PAGE electrophoresis for characterization. Immunological specificity was evaluated using a panel of reference sera. The recombinant OmpH protein from *P. multocida* (serogroup A) was selected as the target marker antigen to develop a universal immunologically based assay system. The preparation of monoclonal antibodies employed standard hybridoma technology using hyperimmune spleen cells from mice fused with mouse myeloma cells. Clones were screened for antibody activity using rec-OmpH antigen. In total, 664 clones exhibited activity. From that group, 30 clones were identified as having high antibody production activity. Next selection steps included the establishment of stable clones and determining the specificity and avidity of antibody activity using ELISA assay systems and Western blot analysis. The antibodies were screen against a panel of 16 strains of *P. multocida* representing strains from groups A, B and D. Eventually, three antibody clones were selected from the final pool of stable clones and transferred to the commercial partner for further evaluation; a sufficient quantity of purified recombinant OmpH antigen was also supplied.

8. The final task was performed by DPRI and the Ivanovsky Virology Institute. The study was directed at determining whether monoclonal antibody reagents developed during Phase I studies against avian influenza and other influenza strains would have demonstrable activity against Type A H1N1 influenza (so called pig influenza virus, PIV) strains and field isolates. With the outbreak of H1N1 influenza and it enhanced transmissibility among humans, birds and swine, similar to the situation noted with highly pathogenic H5N1 avian influenza, that was recognized as being capable of being transmitted between humans and birds and transmitted among birds, it was apparent that there was an immediate need for tools that could rapidly diagnose among these viral infections in different hosts. To accomplish this goal, different H1N1 strains were evaluated based on sequence analysis of the nucleoprotein (NP) gene as seen in in strains from North and South America, Russia and Southeast Asia. Based on these analyses, the NP

gene of a "pig influenza virus" isolate (Type A/Moscow/01/09 (H1N1)) was used to construct the recombinant proteins for this comparison. The NP gene was isolated, sequence, cloned and introduced into plasmid vector for expression in an *E. coli* expression system. The new recombinant NP protein from A/Moscow/01/09 strain is identical to the A/Cal/09/N1H1 strain NP protein. Immuno-reactivity of monoclonal antibodies prepared against avian influenza were tested in Western blot analysis, ELISA assays (direct, indirect and sandwich systems) and lateral flow systems (See Figure 5.). From these studies, the researchers were able to determine that the monoclonal antibodies generated against recombinant and native NP protein specifically interacted with Type A virus preparations from different strains and field isolates, including the pandemic Influenza Virus A (H1N1/Cal/09). The deliverable provided to the commercial partner was appropriate amounts of recombinant NP protein derived from the H1N1 strain and a pair of monoclonal antibodies demonstrating activity against the target H1N1 virus. In preliminary studies evaluating the protective activity of the two antibodies using *in vitro* and *in vivo* assays, a positive response was observed with the monoclonal antibody raised against AIV native H5 hemagglutinin.

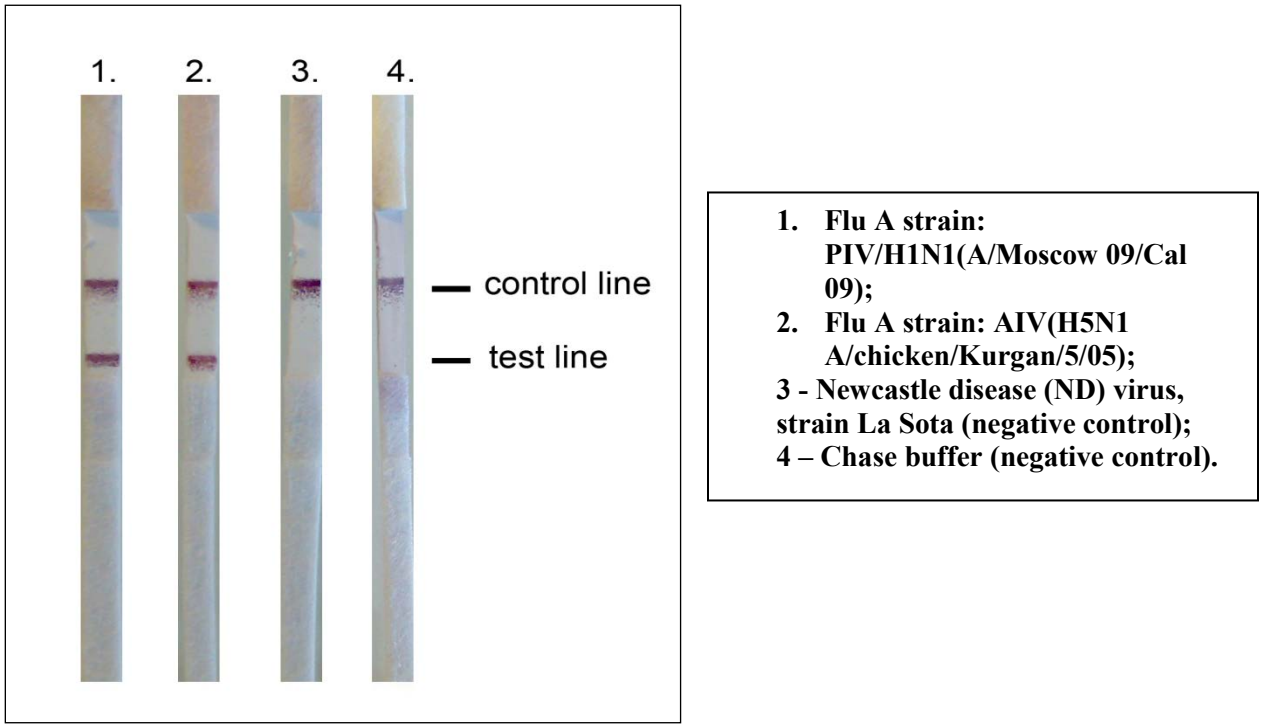



**Figure 5: Detection of Influenza Viruses A (A/H5N1 and A/H1N1) in a Fast Lateral Flow Assay format**

# 3.0 Partners Evaluation of Success

The initial evaluation by New Horizon Diagnostics has been very encouraging and suggests considerable promise for commercialization of products that are developed as a result of work conducted under this CRADA. This is clearly stated in the following comments received at the conclusion of the Phase 1 activities "New Horizon Diagnostics Corp. (NHD) is very pleased with the animal IPP project. The scientific effort and overall cooperation between all FSU groups as well as the US team is exemplary. …. Drs. Sergey Kalnov, Oleg Verkhovsky and team has provided antigens/antibodies as well as technical information with an "eye on the ball" for a commercial product which will meet global needs." Specifically the partner had this to say about activities using avian flu antibodies, "From a commercial – technical point comment, the avian antibodies have been initially reviewed by labs in the US as well as the far East with very good results. The testing will be further expanded to include other countries in the Far East as well as Europe." To provide additional perspective, the commercial partner added this comment: "Given the global threat to food and animal supply as well as an increase in zoonotic diseases, this project is of extreme concern to all, both government and industry. …. Project will achieve the goals of innovative job creation both in USA and the FSU with a needed product/technology, but will also convey in a positive light globally, demonstrating a concern and sensitivity to the issues of human concern." This communication from NHD conveys its considerable appreciation and perspective of success for this GIPP project in product/technology development as well as supporting the global importance for collaborative efforts among all participants.

# 4.0 Future Plans and Commercialization Activities

The plans for commercialization are being pursued on many fronts. This includes the continued demonstration, evaluation and validation of several of the antibody-based diagnostic systems developed under this CRADA. NHD has continued to market these systems as noted in the following correspondence received by PNNL "... the concept of this project is very appealing to not only NHD but also other entities around the world concerned with the threat from mother nature and others which impact food supply, which includes diseases of animals. Consequently, Dr. Ali Mohannadi of WHO, Dr. Tadeusz Wijaszka of the National Veterinary Research Institute (an O.I.E. Reference Center) and others have expressed strong support in these efforts and will assist in validation and utilization of the technologies- this includes poultry as well as swine, cow and other target diseases." The tie to food safety is spelled out clearly in this statement received recently from Dr. Larry Loomis who is the CEO of NHD, "New Horizons Diagnostics has a subsidiary called Safe Food Technology which has great interest in this work."

# 5.0 Inventions as a Result of this CRADA

No new inventions were derived or disclosed under this project.

# 6.0 Conclusion

The CRADA project between NHD and PNNL was highly successful and is generating commercial opportunities in the U.S. and among partner institutes for using the results of this project to develop, manufacture and market diagnostic systems for animal and zoonotic diseases of considerable economic and public health importance. As can be seen by the efforts generated by NHD, the global value of these detection system for human and animal diseases could be considerable in terms of disease detection, prevention, treatment and return on investment and play a key role in developing significant procedures and protocols to detect, monitoring and ultimately control disease outbreaks.

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