# Influenza A H1N1: A Rapid Approach to Cloning and Protein Expression

RP-88


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## ABSTRACT


In May 2009, Swine-derived Influenza A H1N1 virus emerged as a serious threat to global health. With the threat came the need for the virology community to rapidly characterize this emergent subtype. To enable efficient distribution of materials for further characterization, a rapid cloning and protein expression process was implemented using two clinical isolates. Clones were made of all genomic segments/protein coding regions using three different cloning systems. Proteins were expressed in a cell-free system with a HaloTag™ fusion and immobilized on a HaloLink™ microarray substrate. The immobilized proteins were then challenged with anti-H1N1 antibodies to assess immunosensitivity. Mammalian and baculoviral (BEVS) systems were utilized to ensure glycosylation of HA and NA proteins. Clones were publicly available and protein microarrays made within one month after receiving H1N1 DNA.


## OBJECTIVES

- ✦Rapidly respond to influenza A outbreak
- ✦Clone & express H1N1 proteins
- ✦Make clones publicly available in one month
- ✦Fabricate protein microarray

## INTRODUCTION

As new virus variants emerge novel approaches of vaccine development and rapid diagnostic screening are increasingly needed. Inactivated viruses or proteins purified from virus isolates are traditionally used for testing immunogenic response and for vaccine development. This process is time consuming and inefficient and in some cases viruses cannot be cultured at all. Clearly more efficient alternatives are needed. Recombinant protein technologies have the potential to provide a reliable source of antigens for ELISA testing and vaccine development. Once recombinant proteins are selected, then means of expression and production have to be established.

Here we describe rapidly going from sequencing to screening expression systems and generation of recombinant viral proteins using HaloTag™ technology (Promega Corp.) and H1N1 model system. Strains *A/New York/1669/2009(H1N1)* and *A/New York/1682/2009(H1N1)* were sequenced at the Genome Sequencing Center of Infectious Disease (GSCID). All ten influenza CDS's were PCR amplified and cloned using three systems (Gateway®, Ligation Independent Cloning (LIC), Flexi™). Clones were sequence verified and expressed immediately in E.coli (BL21(DE3)Magic) and cell-free wheat germ and subsequently in mammalian and insect cells. Most proteins expressed in all systems and HA successfully secreted from human HEK-293 cells. HaloTag™ was used to immobilize recombinant proteins onto the HaloLink™ slide surface (Promega Corp.) and detected using an anti-HaloTag™ antibody. Influenza proteins were challenged with anti-H1N1 polyclonal antibodies; only matrix protein, M1, demonstrated antibody interaction.

All entry and expression clones were made publicly available on June 5th, 2009 approximately 1 month after strain collection.

## BACKGROUND

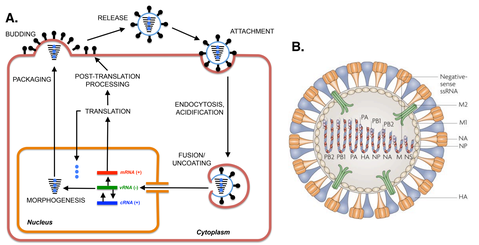


**Figure 1. Overview of Influenza A Virus A)** Influenza virus replication in eukaryotic cells. **B)** Diagram of viral structure(1). Influenza is an enveloped virus containing 8 linear ssRNA(-) genomic pieces coding for 10 proteins. The hypervariable epitopes of the hemagglutinin (HA) and neuraminidase (NA) surface proteins make influenza virus a difficult target for treatment.

## METHODS & MATERIALS

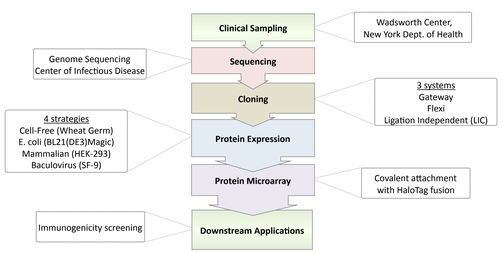


**Figure 2. Project workflow.** Rapid pathway from clinical sampling through sequencing, cloning and recombinant protein expression. Multiple cloning and expression systems employed to ensure high level of success.

| Cloning System | Vector | Fusion Tags | Expression System | Vector Source |
|---|---|---|---|---|
| Gateway | pDONR221 | (entry clone) | - | Invitrogen |
| | GW-cHalo | N: His-Tag, C: HaloTag | Cell Free (SP6), E.coli (T7) | In-house |
| Ligation Independent (LIC) | pLIC-cHalo | C: HaloTag | Cell Free (SP6), E.coli (T7) | In-house |
| Flexi (Promega) | pFC20A | C-term HaloTag | Cell Free (SP6), E.coli (T7) | Promega |
| | pFC14A | C-term HaloTag | Mammalian (CMV) | Promega |
| | pF-BvH1 | N-term HaloTag | Baculoviral (PH) | Promega |
| | pFCBvA | C-term HaloTag | Baculoviral (PH) | In-house |

**Table 1. Cloning and Expression.** Expression clones of 10 influenza A protein coding regions (CDS) were made using Gateway, Ligation Independent cloning (LIC) and Flexi™ (Promega) systems. Gateway entry clones were also made for all 8 genomic segments including untranslated regions (UTR).

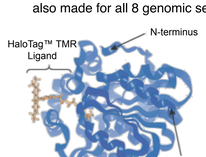


**Figure 3. 3D structure of HaloTag™.** HaloTag™ fusion protein convalently binds the TMR ligand allowing non-reversible binding to resin, solid substrate (HaloLink™ slide) or conjugated fluorophore. The 33kD tag is a modified hydrolase(2).

## RESULTS

### Project Timeline

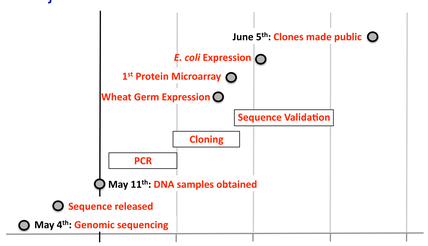


**Figure 4. Project Timeline:** Wadsworth Center at New York Dept. of Health collected strains *A/New York/1669/2009(H1N1)* and *A/New York/1682/2009(H1N1)* and the GSCID at JCVI completed sequencing. Within one month PCR, cloning, expression, sequence validation, and protein microarray were completed. All clones were publicly available on June 5th, 2009

### Cloning/Expression Results

| Target | GW Entry | | *E.coli* / in vitro | | | | | | Mamalian | | Baculovirus | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Seg | CDS | GW-cHalo | | pLIC-Halo | | pFC20A | | pFC14A | | pFBvH1 | | pFCBvA | |
| | C | C | C | E | C | E | C | E | C | E | C | E | C | E |
| PB2 | + | + | + | - | + | + | + | + | + | + | - | N/A | + | N/A |
| PB1 | - | + | + | +(W) | + | + | + | + | + | + | + | - | + | N/A |
| PA | + | + | + | - | + | + | - | N/A | - | N/A | + | N/A | - | N/A |
| HA-ΔTM | + | + | + | - | + | + | + | + | + | +(S) | - | N/A | + | N/A |
| HA(whole) | | + | + | - | | | | | | | | | | N/A |
| NP | + | + | + | - | - | N/A | - | N/A | + | - | - | N/A | + | N/A |
| NA-ΔTM | + | + | + | - | + | + | + | + | + | +(N) | + | - | + | N/A |
| NA(whole) | | + | + | - | | | | | | | | | | N/A |
| M1 | + | + | + | - | + | + | + | + | + | + | + | + | + | N/A |
| M2 | | + | + | - | + | + | + | N/A | + | + | + | N/A | + | N/A |
| NS1 | | + | + | - | + | + | + | N/A | + | + | + | N/A | + | N/A |
| NS2 | + | + | + | - | + | + | + | + | + | + | + | N/A | + | N/A |
| Total | 8 | 12 | 12 | 1 | 9 | 9 | 8 | 6 | 9 | 8 | 7 | 1 | 9 | N/A |

**Figure 5. Clone/Expression Tally:** Results of Gateway, LIC, and Flexi™ cloning and multiple expression systems. Removed transmembrane domains (HA & NA) to enhance solubility and secretion. Overall 20 entry clones and 54 expression clones were created and sequence verified. Most proteins cloned and expressed consistently; the NP protein could not be expressed. (C=cloning, E=Expression, W=Wheat germ only, S=secreted, N=not secreted)

### Protein Microarray

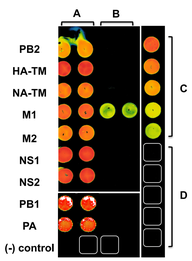


**Figure 6. On-Chip purification** using Halolink™ slides and gaskets. E. coli cell supernatants containing Halo-fusion recombinant proteins were deposited in the gasketed wells. Excess sample and E. coli endogenous proteins were washed off with PBSI. Primary antibodies (**A**. Rabbit anti-HaloTag or **B**. Goat anti-H1N1 virions) were incubated, washed and followed by a secondary antibody incubation (**A**. Goat anti-Rabbit_alexa647 or **B**. Chicken anti-Goat_alexa647). *E. coli* supernatant was used as a negative control. Slides were scanned (Axon 4000B) following a final PBSI wash. Spot size is 3.3 mm. **C.** HaloTag standards (conc. 2.5 ug/uL,1.25ug/uL, 625ng/ul, 313ng/ul, 156 ng/ul.) detected with anti-HaloTag antibody **D.** HaloTag standards showing no specificity with anti-H1N1 polyclonal antibodies.

### Mammalian expression

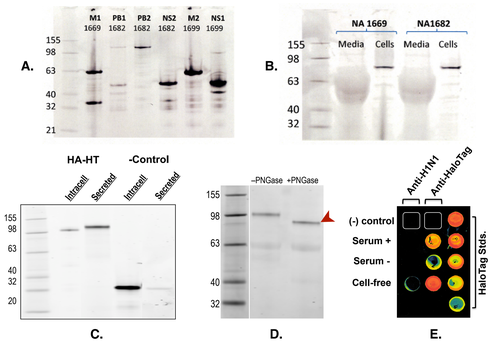


**Figure 7. Protein Expression in Human Cell Line:** Cells were collected and lysed in SDS and equivalent fractions run on a SDS-PAGE gel. **A)** Human HEK-293 cells transfected with pFC14A clones. Harvested after 24 hours transient expression and stained with TMR ligand. **B)** Neuraminidase (NA) expressed intracellularly but failed to secrete. **C)** At 48 hours after transfection 3 times more HA-HaloTag fusion protein is secreted vs. intracellular; the unfused HaloTag negative control was not secreted. **D)** A down-shift in molecular weight after PNGase treatment suggests secreted HA is glycosylated (red arrow). **E.** On-chip purification of secreted HA on HaloLink™ slide; no specific interaction with Anti-H1N1 antibodies observed.

### Baculoviral expression

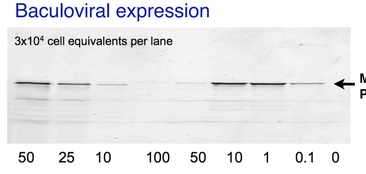


**Figure 8. Viral titer determination:** Sf9 insect cells were infected with different amounts of passage 2 (P2) virus containing Matrix protein 1 (M1) and labeled with TMR. Cells was collected in 100ul of storage buffer and 15ul of the sample was loaded on the gel. Estimated expression level: ~ 3.50 ug/ 2x10⁶cells/1ml when compared to GST-HaloTag reference.

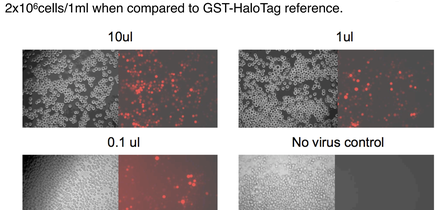


**Figure 9. In vivo Expression Levels:** Titration of P2 viral stock of Matrix protien (M1) stained with TMR ligand. Comparison of phase contrast microscopy with fluorescence microscopy.

## DISCUSSION

In early May, 2009 we were challenged to produce and distribute clones for two emergent strains of Influenza A H1N1. We quickly made 20 Gateway® compatible entry clones, and 54 expression clones. The nucleoprotein (NP) was the most difficult target to PCR/clone using the LIC and Flexi methods. Within a month sequence verified clones were publicly available. Within this first month we expressed protein in E. coli and cell-free wheat germ and fabricated an assayable protein microarray. Within two months most of the ten proteins were transiently expressed in mammalian cells and one protein in insect cells.

HaloTag™ fusion allowed direct spotting of crude cell lysates on the protein microarray. The HaloTag covalently bound the ligand coated microscope slide and thus enabled on-chip purification with minimal background signal. By eliminating the protein purification step the scale of production and prep time was significantly reduced.

Surface exposed hemagglutinin (HA) and neuraminidase (NA) proteins are important for assaying immunosensitivity. We successfully secreted the HA-HaloTag fusion from human HEK-293 cells and observed a molecular weight down-shift with PNGase treatment suggesting HA glycosylation. The NA protein expressed intracellularly but was not secreted. Only Matrix protein (M1) showed sensitivity when the protein microarray was challenged with anti-H1N1 antibodies.

## FUTURE DIRECTIONS

- ✦ Expand comparison to include more strains
- ✦ Alternate NA constructs for better secretion
- ✦ Comprehensive microarray comparison of proteins from all expression systems

## REFERENCES


(1) Nelson M.I., Holmes E.C., **The evolution of epidemic influenza**. (2007) *Nat Rev Gen 8: 196-205.*

(2) Ohana R.F., Encell L.P., et al. **HaloTag7: A genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification.** (2009) Prot Exp Pur 68: 110-120.

(3) Schwarzer J., Rapp E., et al. **Glycan analysis in cell culture-based influenza vaccine production: Influence of host cell line and virus strain on the glycosylation pattern of viral hemagglutinin.** (2009) *Vaccine* 27: 4325-4336.

(4) Xu X., Zhu X., **Structural characterization of the 1918 influenza virus H1N1 Neuraminidase.** (2008) *J of Virology* 82 (21):10493-10501

(5) Jabbar M., Nayak D.P., **Signal processing, glycosylation, and secretion of mutant hemagglutinins of human influenza virus by S. cerevisiae.** (1987) *Mol Cel Biol,* 7 (4): 1476-1485.


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Clones Available at: http://pfgrc.jcvi.org/fir_index.php/fir/available_organisms.html