Single-Cell Cloning of H5N1 HA-Transfected CHO Cells & Protein Expression Analysis

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Abstract

Avian influenza, caused by influenza Type A virus, poses significant threats to both animal and human health worldwide. Among these, the highly-pathogenic H5N1 subtype has emerged as a critical concern, causing severe global outbreaks in poultry, substantial economic loss, and ongoing zoonotic infections in humans[2]. In this study, we generated recombinant H5N1 proteins through single-cell cloning of transfected Chinese Hamster Ovary (CHO) cells to verify protein expression. We utilized plasmids encoding influenza H5N1 protein (strain B3.13 with Bovine IgG1) and His-tagged control proteins from bovine (strain B3.13) and human (strain D1.1) origins. Cells were transiently transfected using PEI MAX reagent and subsequently expanded in single-cell-derived clones. Protein expression was screened and confirmed via dot blot and Western blot analyses. Our findings demonstrate a robust methodological framework to produce stable recombinant influenza proteins, essential for further research toward diagnostics, therapeutics, and vaccine development.

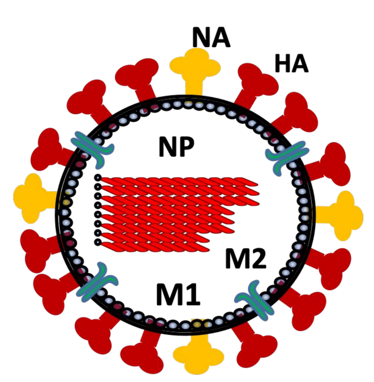

Figure 1. Schematic Model of Influenza A Virus
Influenza A viruses are categorized through two major surface glycoproteins: hemagglutinin (HA) with 16 known subtypes (H1–H16), and neuraminidase (NA) with 9 subtypes (N1–N9)[1].

Methods

- **Cell Culture & Transfection:** CHO cells were seeded in 6-well plates at a density of $2{\times}10^6$ cells/well and cultured for 18 hours. Cells were transiently transfected with 2 µg of pDNA encoding either H5N1 protein + B3.13-BoIgG1, bovine His-tagged control protein (B3.13-His), or human His-tagged control protein (D1.1-His). Transfections were done using polyethylenimine (PEI) Max at a DNA:PEI ratio of 1:6, according to standard protocols.
- **Single-Cell Cloning:** Cells were single-cell cloned manually into 96-well plates to ensure monoclonality. Cells were maintained under standard culture conditions until sufficient confluency was achieved.

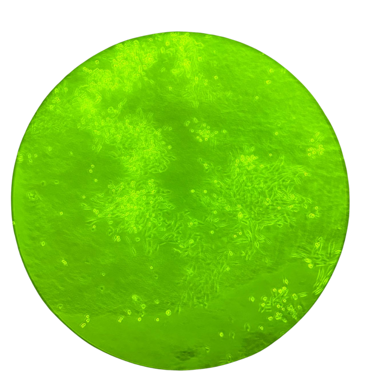

Figure 2. CHO cell in suspension culture. Brightfield image of CHO cells in media, representative of cells isolated during single-cell cloning. Cells were manually isolated and plated into 96-well plates to establish monoclonal populations.

- **Protein Expression Analysis (Dot Blot) & Further Confirmation by Western Blot:** His-tagged control proteins (B3.13-His and D1.1-His) were probed overnight at 4°C with biotin-conjugated anti-His primary antibody (1:10,000 dilution in blocking buffer). Membranes were incubated with HRP-conjugated streptavidin secondary antibody (1:20,000 dilution in 3% BSA) for 2 hours at room temperature. H5N1 fusion protein (B3.13 - BoIgG1) was blocked with 5% milk in PBST and probed overnight at 4°C with HRP-conjugated bovine IgG antibody (1:10,000 dilution). No additional secondary antibody was required. Membranes developed using Immobilon® ECL UltraPlus Western HRP Substrate.

Results

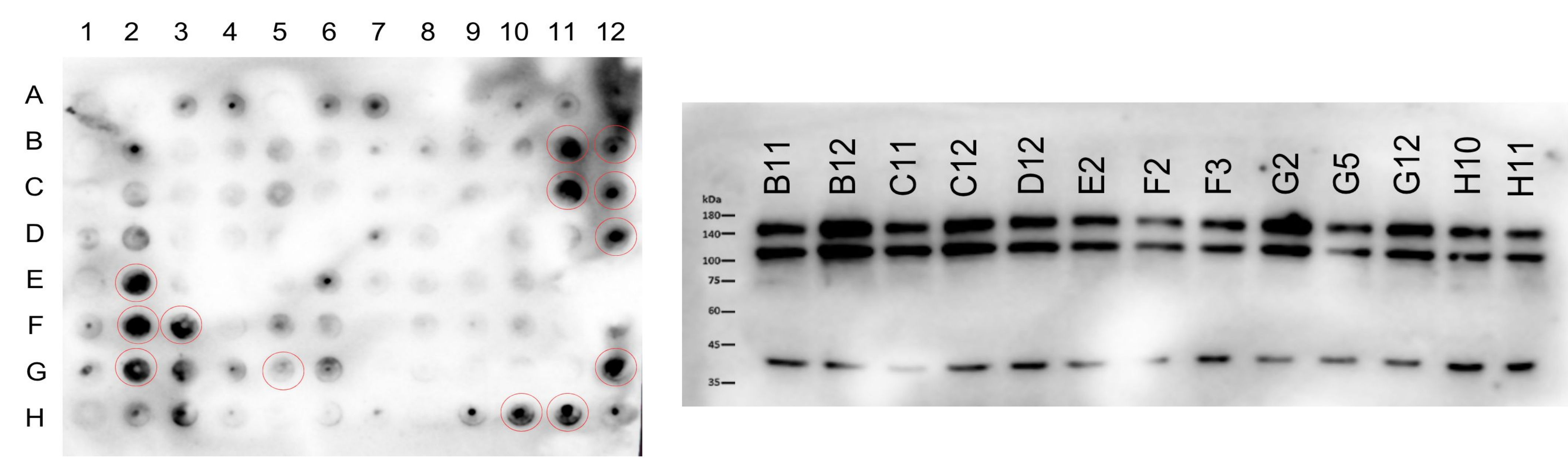

Figure 3. Screening and validation of H5N1: B3.13 BoIgG1 transfected CHO cells
(Left) Dot blot screening of single-cell CHO clones expressing recombinant H5N1 protein (B3.13-BoIgG1). Cell supernatants from 96-well plates were probed with HRP conjugated BoIgG, followed by chemiluminescence detection. Positive dot-blot clones assayed for Western blot are circled in red. Rows and columns line up to the original 96-well plate layout.
(Right) Western blot validation of selected positive clones from the dot blot screening. Lysates were resolved on 10% gel through SDS-PAGE and probed for expression of the ~115 kDa H5N1 fusion protein using HRP-conjugated BoIgG antibody and PM2500 ladder. Lanes labeled according to well positions (e.g., B11, C11, G2) show consistent expression of the target protein across clones.

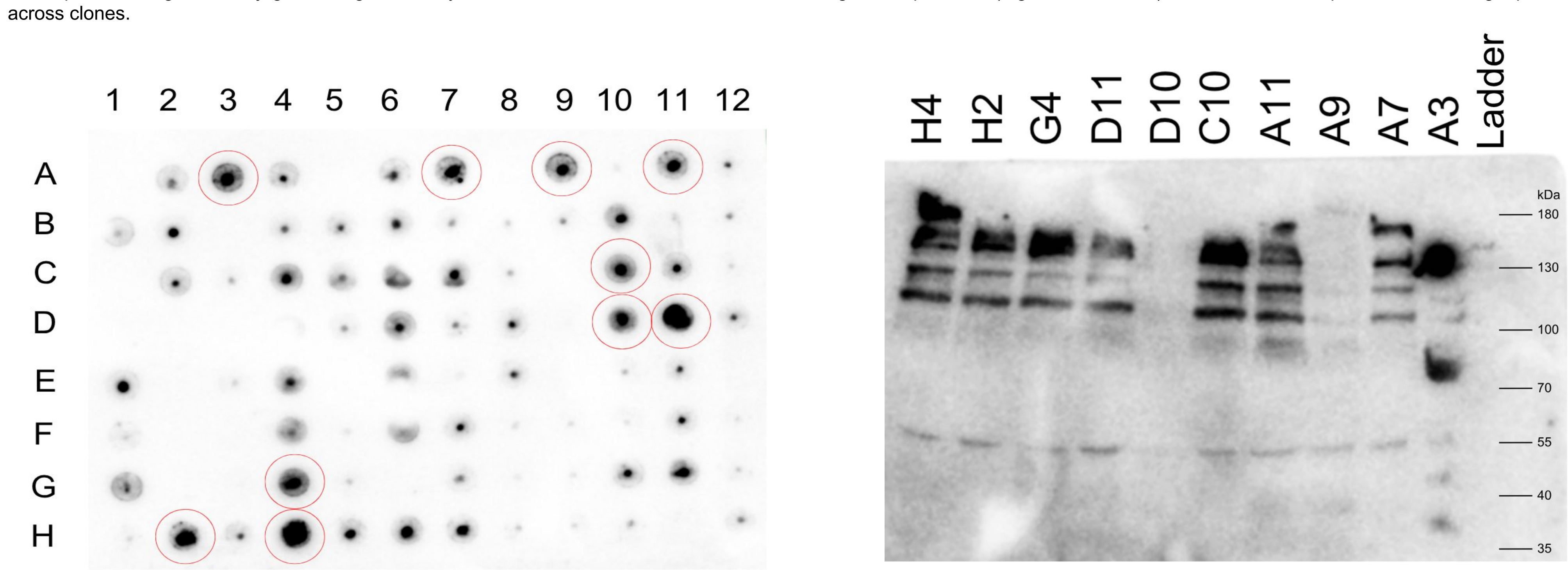

Figure 4. Screening and validation of H5N1: B3.13 His transfected CHO cells
(Left) Dot blot screening of single-cell-derived CHO clones expressing recombinant H5N1 protein (B3.13-His). Cell supernatants from 96-well plates were probed biotin conjugated anti-His primary antibody (1:10,000) overnight. HRP Streptavidin secondary antibody (1:20,000) was applied for 2 hours in 3% BSA.
(Right) Western blot validation of selected positive clones from the dot blot screening. Supernatants were resolved on 10% gel through SDS-PAGE and probed for expression of the 68 kDa H5N1-His fusion protein using PageRuler™ Prestained Protein Ladder (10 to 180 kDa). Multiple clones show distinct bands around the expected size. Lane A3 shows unexpected ladder-like bands around the 60 kDa and 45 kDa markers, likely due to contamination from the adjacent ladder well.

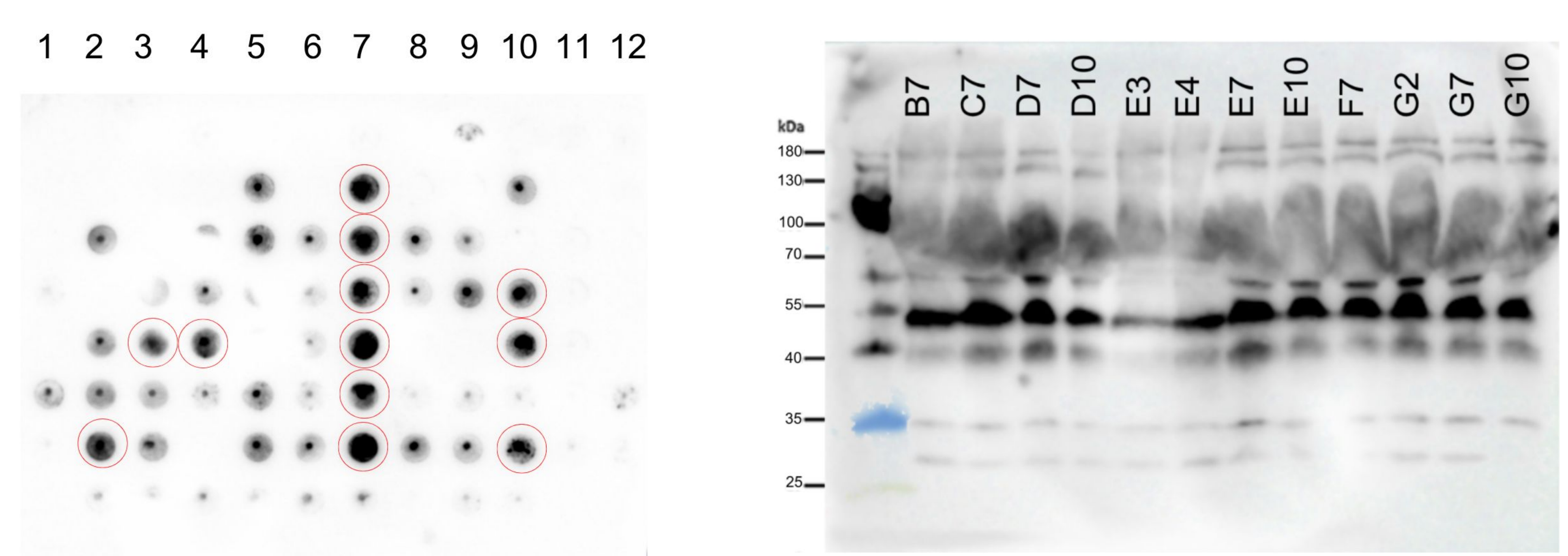

Figure 5. Screening and validation of H5N1: D1.1 His transfected CHO cells
(Left) Dot blot analysis of CHO clone supernatants transfected with the D1.1 plasmid. Samples were treated overnight with biotin-conjugated anti-His antibody (1:10,000), followed by HRP-conjugated streptavidin secondary antibody (1:20,000) in 3% BSA for 2 hours. Clones showing strong signals are circled, with several high-expressing clones observed in column 7.
(Right) Western blot analysis of selected dot blot-positive supernatants. Supernatants were resolved on 10% gel through SDS-PAGE and probed with the same anti-His/streptavidin detection system. Recurring band(s) at 68 kDa is observed across multiple clones, likely corresponding to the expected molecular weight of the D1.1 His-tagged protein. The presence of multiple bands and background signal indicates non-specific binding or lysate complexity. Smearing may be a result of residual genomic DNA, incomplete denaturation, or overloading.

Conclusions

We successfully generated and screened CHO cell clones expressing recombinant H5N1 (B3.13-BoIgG1) and His-tagged control proteins (B3.13-His and D1.1-His). Dot blot analysis allowed us to identify high-expressing clones, and Western blotting ruled out false positives and confirmed protein expression at the expected molecular weights of 115 kDa for H5N1-BoIgG and 68 kDa for both H5N1-His B.3.13 and D.1.1.

Overall results between positive samples were consistent, but some Western blots showed minor background signal and additional bands, which was likely due to sample complexity or residual nucleic acids within. We aim to enhance the clarity of future samples by optimizing lysate preparation through sonication, refining antibody dilutions, and incorporating stronger washing reagents to minimize non-specific binding. Additionally, the presence of heavier bands in some clones could represent the glycosylated forms or protein dimers of our proteins, which we can aim to resolve by running a non-reducing SDS-PAGE.

These improvements to our methodology will allow us to ensure accurate protein production and deeply analyze the structure and function of key H5N1 antigens such as FcRn, β2m, and FcγR1. Since H5N1 remains a serious concern, this work is imperative for future applications such as testing immune responses, identifying key antigen regions, and developing more precise and effective vaccines and diagnostic tools against avian influenza.

Acknowledgement

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Citations

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