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Use of an Influenza Antigen Microarray to Measure the Breadth of Serum Antibodies Across Virus Subtypes

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TITLE:

Use of an Influenza Antigen Microarray to Measure the Breadth of Serum Antibodies Across Virus Subtypes

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KEYWORDS:

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SUMMARY:

We present a protocol for using a protein microarray constructed by printing influenza antigens onto nitrocellulose-coated slides to simultaneously probe serum for multiple antibody isotypes against over 250 antigens from different virus strains, thus allowing measurement of the breadth of serum antibodies across virus subtypes.

ABSTRACT:
The influenza virus remains a significant cause of mortality worldwide due to the limited effectiveness of currently available vaccines. A key challenge to the development of universal influenza vaccines is high antigenic diversity resulting from antigenic drift. Overcoming this challenge requires novel research tools to measure the breadth of serum antibodies directed against many virus strains across different antigenic subtypes. Here, we present a protocol for analyzing the breadth of serum antibodies against diverse influenza virus strains using a protein microarray of influenza antigens.

This influenza antigen microarray is constructed by printing purified hemagglutinin and neuraminidase antigens onto a nitrocellulose-coated membrane using a microarray printer. Human sera are incubated on the microarray to bind antibodies against the influenza antigens. Quantum-dot-conjugated secondary antibodies are used to simultaneously detect IgG and IgA antibodies binding to each antigen on the microarray. Quantitative antibody binding is measured as fluorescence intensity using a portable imager. Representative results are shown to demonstrate assay reproducibility in measuring subtype-specific and cross-reactive influenza antibodies in human sera.

Compared to traditional methods such as ELISA, the influenza antigen microarray provides a high throughput multiplexed approach capable of testing hundreds of sera for multiple antibody isotypes against hundreds of antigens in a short time frame, and thus has applications in sero-surveillance and vaccine development. A limitation is the inability to distinguish binding antibodies from neutralizing antibodies.

INTRODUCTION:
The influenza virus is responsible for a loss of 20 million life-years annually by death or disability, including 1% of all deaths worldwide each year, with disproportionate impacts on the elderly and populations in the tropics and developing world[1-3]. In addition to the disease burden of seasonal epidemics, the emergence of novel influenza strains via genetic re-assortment either naturally in common hosts or artificially for bioterrorism could lead to worldwide pandemics with rapid spread and high lethality[4,5]. While numerous influenza vaccines are currently available, their effectiveness is limited by subtype specificity[6], creating the need to develop universal influenza vaccines that confer long-lasting immunity against multiple virus strains[7].

A key challenge to the development of universal influenza vaccines is high antigenic diversity across strains. The antigenic specificity of current vaccines combined with antigenic variation of circulating viruses creates a mismatch between vaccine strains and circulating strains. This confers an evolutionary advantage favoring further genetic drift away from vaccine strains during an epidemic, limiting vaccine efficacy often to less than 50%[8,9]. An additional source of antigenic mismatch is egg-adaptive viral mutations generated during vaccine manufacture, which lead to antibodies that bind poorly to circulating viruses[10,11].

Overcoming this challenge of high antigenic diversity will require novel research tools to

characterize the breadth of pre-existing and elicited immune responses across clinically relevant antigenic variants in serum and mucosal specimens. Currently available methods, including hemagglutination inhibition (HAI), microneutralization (MN), and traditional ELISA, are limited to detecting antibodies against a single virus strain at a time, so their use for detection of multiple antibody isotypes against multiple virus strains quickly exhausts available clinical specimen and laboratory resources. Furthermore, HAI and MN require live virus culture that is only available in specialized laboratories.

Protein microarrays, potentially consisting of up to thousands of antigens printed onto nitrocellulose-coated slides as shown in **Figure 1**, can fill this need[12]. These microarrays can be produced and probed in a high throughput manner while consuming small quantities of clinical specimen to determine quantitative antibody isotype/subtype levels against each individual antigen on the array. This approach to antigen discovery has been applied to diagnostic and vaccine development against multiple infectious pathogens[13]. To date, we have produced protein microarrays for over 35 pathogens including over 60,000 total expressed proteins and used them to probe over 30,000 human sera from infected and control individuals. A recently developed portable imaging platform for microarray slides has made this methodology more accessible to the end user[14].

Building on extensive previous work by multiple contributors in the field[15-19], an influenza protein microarray was recently developed that contains over 250 purified hemagglutinin (HA) antigenic variants with representation of all 18 subtypes[12,20]. Using this methodology, a natural influenza infection was demonstrated to generate broadly reactive IgG and IgA antibodies against phylogenetically related HA subtypes, while an intramuscular influenza vaccination generated only subtype-specific IgG antibodies[21]. However, adding an adjuvant that activates toll-like receptors to influenza vaccines was shown to broaden the elicited IgG antibody response across HA subtypes in animal studies[22].

This microarray is currently being used to probe sera collected from a prospective cohort study of college students who were followed for influenza infection. Here, the methodology of the influenza antigen microarray with demonstration of assay reproducibility to detect subtype-specific and cross-reactive antibodies in a subset of specimens from this study is reported.

PROTOCOL:

All human sera are handled and disposed according to approved institutional protocols for biosafety with use of protective personal equipment. All laboratory personnel participating in this protocol have received training in biosafety and research ethics.

1. Produce influenza antigen microarrays

1.1. Design and obtain antigen set for microarray

1.1.1. Obtain expressed and purified protein antigens as lyophilized powder.

1.2. Print antigens onto microarray slides

1.2.1. Reconstitute each lyophilized antigen to a concentration of 0.1 mg/mL in phosphate-buffered saline (PBS) with 0.001% Tween-20 (T-PBS). Transfer 10 µL of each reconstituted antigen to individual wells of an untreated 384-well flat-bottom plate.

1.2.2. Print antigens using a microarray printer (the microarray printer used in this study is no longer commercially available, see **Discussion**) with low-volume microarray spotting pins that aspirate antigen into the sample channel and deposit via direct contact and capillary action onto 16-pad nitrocellulose-coated glass slides.

1.2.2.1. Program the printing software (e.g., Gridder) with the source plate configuration and printing parameters.

1.2.2.1.1. Use the pull-down menu to select the name of the plate type that will be used with this printing method. For this study, use an untreated 384-well flat-bottom plate.

1.2.2.1.2. Select the text box next to **Number of Plates** and type the number of sample plates that will be used in this printing protocol. For this study, use 1 plate.

1.2.2.1.3. Select a pin configuration to use with this method. For this study, use an 8-pin configuration.

1.2.2.1.4. Ensure the origin offsets are the distances (in the X- and Y-directions) between the slide origin (which is calibrated in the Administrative section) and the location where the printing pins will start printing on the slides.

1.2.2.1.5. Using the **Array Design** tab, define the size and shape of the arrays (dot spacing and number of dots per subarray). For this study, print 324 spots (180 µm diameter with 300 µm spacing) onto 16-pad slides in an 18x18 format using 8 pins.

1.2.2.1.6. Select the parameters for how the printing pins pick up and dispense samples. For this study, each pin aspirates 250 nL of antigen solution and prints 1 nL onto each of 40 spots (total of 20 slides for all 8 pins)

1.2.2.1.7. Configure the pin cleaning protocol and blotting protocol. For this study, each pin prints antigen, is dipped in sterile ddH_2O in sonicated wash container, and then aspirates next antigen.

1.2.2.1.8. Define the sequence in which the sample blocks are printed onto the slides. The array software will construct an annotated grid index (.gal) file to describe the arrangement of antigens within each microarray.

NOTE: The top row is used for fiducials (with fluorescence at all wavelengths used in imaging, e.g., mix of Qdot 585 nm streptavidin conjugate and Qdot 800 nm streptavidin conjugate in this study) to orient grids during imaging. For long printing runs, antigens in source plate can be periodically re-suspended by pipetting up and down and then centrifuging, or new source plate can be prepared and used.

1.2.3. Place un-probed microarray slides in a lightproof box and keep in a desiccator cabinet at room temperature for long-term storage.

NOTE: The protocol may be paused indefinitely at this point.

1.3. Perform quality control check (requires poly-histidine tags)

1.3.1. Attach the slide to probing chambers and rehydrate with blocking buffer as described in step 2.1.1-2.1.2 and shown in **Figure 2**.

1.3.2. Dilute the mouse monoclonal poly-His antibody 1:100 in filtered 1x blocking buffer.

NOTE: If non-purified protein antigens (e.g., expressed in in vitro transcription and translation system) are directly used to print microarrays, add components of the protein expression system (e.g., *E. coli* lysate) in a ratio of 1:10 with the blocking buffer used for serum dilution in order to block any antibodies directed against these components.

1.3.3. Add 100 µL of diluted poly-His antibody to each slide chamber containing array pad after aspiration and incubate for 2 h at room temperature or overnight at 4 °C on shaker. Wash 3x with T-TBS buffer as described in step 2.2.1. Dilute biotin-conjugated goat anti-mouse IgG secondary antibody 1:200 in blocking buffer, add 100 µL per well after aspiration, and incubate for 1 h at room temperature on shaker. Wash 3x with T-TBS buffer.

1.3.4. Dilute Qdot 585 nm streptavidin conjugate to 4 nM in blocking buffer, add 100 µL per well after aspiration, and incubate for 1 h at room temperature on shaker. Wash 3x with T-TBS buffer, and then once with TBS buffer (without Tween).

1.3.5. Dissemble and quantify slides as described in step 2.2.5 – 3.1.2.

NOTE: Protein antigens must contain His_{10} tags to use this quality control protocol. Alternatively, if a different tag is included, quality control check can be performed with antibody or ligand for that tag.

2. Probe sera for influenza antibodies using microarrays

2.1. Incubate sera on microarrays for antibody binding

2.1.1. Attach microarray slides to chambers using clips and place in frames as shown in **Figure**

3.

NOTE: Always avoid touching the microarray pad with hands and instruments. Ensure that slides are oriented with the pad side up and the small notch in upper right corner.

2.1.2. Rehydrate microarray slides with 100 µL per well of filtered 1x blocking buffer, and dilute serum 1:100 in 100 µL of blocking buffer (can use untreated 96-well plates or 2 mL tubes). Incubate both rehydrated microarray slides in covered frames and diluted sera separately for 30 minutes at room temperature on orbital shaker at 100-250 rpm. Perform all subsequent incubation steps similarly on the shaker.

NOTE: Sera should be aliquoted and frozen at -80 °C for long-term storage to minimize freeze-thaw cycles and should be vortexed to mix and centrifuged to remove particulates prior to use. Observe slide chambers carefully during and after this step to detect any leakage that requires re-assembling slide chambers.

2.1.3. Using pipette tips connected to a vacuum line with secondary collection flask, carefully aspirate blocking buffer from corner of each chamber without touching pads. Perform all subsequent aspiration steps similarly. Add diluted sera to pads quickly after aspiration in order to not allow pads to dry.

2.1.4. Place covered frames in trays inside a secondary container surrounded by moist paper towels and sealed to prevent evaporation. Incubate overnight at 4 °C on rocking shaker (alternatively, can incubate for 2 h at room temperature on orbital shaker at 100-250 rpm).

2.2. Label bound serum antibodies with quantum-dot-conjugated secondary antibodies

2.2.1. Aspirate sera from chambers carefully as described above, add 100 µL per well of T-TBS buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 in ddH_2O adjusted to pH 7.5 and filtered, can be obtained commercially), and incubate for 5 min on orbital shaker at 100-250 rpm. Repeat this wash step a total of 3x (all subsequent wash steps are performed similarly).

2.2.2. Prepare mixture of secondary antibodies diluted to 1 µM in blocking buffer and mix thoroughly by pipetting prior to and during use to maintain homogeneity.

NOTE: To maintain assay reproducibility, the same batch of each secondary antibody should be used for all probing experiments for which quantitative comparison of data across experiments is planned. The specific concentration of secondary antibody may need to be varied depending on the affinity; follow manufacturer's protocols whenever available.

2.2.3. Aspirate buffer from chambers after final wash, add 100 µL per well of secondary antibody mixture, and incubate for 2 h at room temperature on shaker.

2.2.4. Aspirate secondary antibody mixture wash 3x with T-TBS buffer, and then wash once

with TBS buffer (without Tween).

2.2.5. Dissemble microarray slides from chambers carefully to avoid touching pads, rinse gently with filtered ddH_2O, and dry by placing in 50 mL tubes and centrifuging at 500 *x g* for 10 min.

2.2.6. Place probed microarray slides in a lightproof box and keep in a desiccator cabinet at room temperature for long-term storage.

NOTE: The protocol may be paused for up to 1 week at this point.

3. Quantify antibody binding to antigens within microarray

3.1. Visualize microarray slides and quantify spot fluorescence intensity to measure antibody binding

3.1.1. Acquire images of microarray slides using the portable imager with built-in software.

3.1.1.1. In the **Configure Imager** tab, select the proper slide configuration. For this study, use 16-pad slides.

3.1.1.2. In the **Image Control** tab, select the proper fluorescent channel, and adjust the gain, exposure time, and acquisition time depending on the reactivity of the sera to obtain optimal images. For this study, the fluorescent channels for IgA and IgG were 585 nm and 800 nm respectively, and imaging settings were gain of 50, exposure time of 500 ms, and acquisition time of 1 s.

3.1.1.3. Click on **Capture** to start the process of acquiring the image.

NOTE: Microarray slides can be re-imaged at multiple settings without degradation of signal as long as they are stored dark and dry. Other imaging systems can be used if compatible with the slides.

3.1.2. Detect array spots using grids oriented based on the fiducial markers and measure spot intensity as median of pixel intensity minus background measured around spots. Perform this quantification algorithm in batch using the built-in imager software, which utilizes the .gal file constructed in step 1.2.2 to connect spot intensities to individual antigens on each microarray.

3.1.2.1. In the **File Info** panel, upload the .gal file by selecting from its folder on the computer, and specify the folder where the analysis output files are to be saved in the **Analysis Options** section.

3.1.2.2. In the **Image Control** tab, open one of the acquired images to be quantified and select the **Auto** button in the upper right corner.

3.1.2.3. In the **Array Analysis** section in the lower right corner, create a fiducial template as instructed by the software.

3.1.2.4. Click on the **Batch Analysis**, select the folder that contains the images to be quantified, and select the fiducial template that was created in the previous step. The software analyzes each image and quantifies the spot intensity.

NOTE: This step will generate a .csv file containing spot intensities quantifying antibodies within each serum specimen that bind to each individual antigen on the microarray that can subsequently be manipulated in spreadsheet manipulation or analysis software.

3.1.3. Analyze raw data to compare antibody binding across antigens and across serum specimens. For this study, IgA and IgG antibodies measured as 585 nm and 800 nm fluorescent spot intensities were compared across all antigens between 2 independent runs of the experiment using different slides on different days, and correlation analysis was performed to measure assay reproducibility.

NOTE: For non-purified proteins printed as expression mixture, data analysis should begin with background subtraction of a no DNA control.

REPRESENTATIVE RESULTS:

As a demonstration of the protocol, baseline sera were assayed from 16 individuals within a prospective cohort study of college students followed for influenza infection on the influenza antigen microarray. To demonstrate assay reproducibility, these specimens were probed twice, on different slides and different days.

For this study, purified influenza antigens containing His_{10} tags were obtained from commercial vendor (see **Table of Materials**) and collaborators. These antigens include 251 total HA antigens, with 63 globular head domains (HA1) and 186 full-length proteins (HA0), including 96 monomeric HA0 proteins and 90 trimerized HA0 proteins containing fused trimerization ("foldon") domain[23]. A full list of antigens and controls used in this study is included as a **Supplementary File**. For this study, secondary antibodies used were goat anti-human IgG conjugated to quantum dot emitting at 800 nm (GAH-IgG-Q800) and goat anti-human IgA conjugated to quantum dot emitting at 585 nm (GAH-IgA-Q585) for multiplex detection of IgG and IgA antibodies as shown in **Figure 3**. IgG and IgA antibody binding to different subtypes and molecular forms of influenza HA antigens were compared for sera obtained from the clinical cohort described above.

The resulting heat map is shown in **Figure 4** with graphical representation in **Figure 5**. In these figures, only the clinically relevant subtypes with high representation on the array are labeled to save space, with "+" denoting all remaining subtypes of higher number, and minor or less well represented subtypes included as ordered (e.g., H2 in between H1 and H3). A full list of strains and subtypes on the array is included as **Supplementary File**. These data demonstrate

that antibodies to the head group of HA are subtype-specific with expected high quantity of antibodies to clinically prevalent strains (H1N1, H3N2, and B) and low quantity of antibodies to other strains. However, antibodies to the whole HA, which includes the stalk domain, are more cross-reactive across subtypes, and this effect appears to be augmented when the whole HA is trimerized. This result is not unexpected, as the whole HA includes the stem region, which is highly conserved across subtypes. Therefore, antibodies to whole HA molecules from non-clinical subtypes (e.g., H5 and H7) likely represent anti-stem antibodies originally elicited against clinical subtypes (e.g., H1 and H3) that are cross-reacting with the stem regions of the other HA subtypes on the array. This point illustrates the importance of including both head HA and whole molecule HA on the array to distinguish between antibodies to the head and the stem regions which show different reactivity profiles.

The assay demonstrates good reproducibility across probing runs. The second run does show slightly lower IgG antibodies across all strains, although the pattern between the strains is consistent. This across-the-board slight decrease is likely due to batch-to-batch variability in the secondary antibody, which was changed between runs for IgG but not for IgA. Thus, as noted in the protocol, it is recommended to use the same batch of each secondary antibody for any experiments between which quantitative comparison is planned. If different batches of antibody are necessary due to a high number of samples to be tested, we recommended including shared samples between experimental runs with different antibody batches to allow for quantitative comparison with correction.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of protein microarray. Each slide contains multiple pads each with a single array, which consists of hundreds of antigens printed onto spots arranged in a grid, with each spot containing one antigen adsorbed onto the 3-dimensional topography of the nitrocellulose surface to which antibodies from serum are bound.

Figure 2: **Schematic of influenza antigen microarray printing and probing protocol.** From left to right, microarray is printed using onto nitrocellulose-coated slides, which are used to probe sera for IgG and IgA antibodies using quantum-dot-conjugated secondary antibodies, with slides imaged using a portable imager, and results analyzed to generate a heat map.

Figure 3: Procedure for attaching probing chamber to microarray slide. From **A** to **F**, the probing chamber is placed on top of slide in correct orientation, attached to the slide using horizontal clips on the sides, and placed in the probing tray.

Figure 4: Representative results of influenza antigen microarray. Heat maps represent antigen-specific antibody responses, with each row representing a single antigen arranged by molecule, subtype, and strain, and each column representing a probing run of a single specimen, arranged by antibody isotype and run (**A**, white = 0, black = 20000, red = 40000 fluorescence intensity). The antigen subtypes including all hemagglutinin subtypes from 1 to 18 and all neuraminidase subtypes from 1 to 10 are arranged vertically and labeled on the left. A comparison of the fluorescence intensity between two runs demonstrates good assay

reproducibility by linear regression for IgA (**B**) and IgG(**C**).

Figure 5: **Breadth of serum antibodies measured on influenza antigen microarray.** Serum IgA (**A**) and IgG (**B**) are grouped by HA and NA molecular forms and subtypes to demonstrate high specificity of HA head group antibodies for clinical subtypes and high cross-reactivity of whole HA and trimerized whole HA antibodies with inclusion of stalk region.

Supplementary File: **List of antigens on influenza antigen microarray.** Content is shown for all 324 spots on the array, including blanks, fiducials, controls (human IgG and IgA and anti-human IgG and IgA at 0.1 mg/mL and 0.3 mg/mL), and antigens with information on source, molecular form, subtype, and strain. Abbreviations are as follows: for source, Sino = Sino Biological Inc., FKL = Florian Krammer Laboratory; for molecular form, HA1 = head HA, HA0 = whole HA, HA2 = stalk HA, NP = nucleoprotein. For antigens sourced from Sino Biological Inc., catalog numbers are shown; for antigens sourced from Krammer Laboratory, antigen IDs are listed.

DISCUSSION:

The influenza antigen microarray protocol described here is adaptable to any project that requires analyzing antibody responses to many antigens. The microarray platform can be used with any desired set of protein antigens expressed in any system that can achieve 0.1 mg/mL or higher yield with or without purification as previously described[12]. If non-purified protein antigens (e.g., expressed in in vitro transcription and translation system) are directly used to print microarrays, components of protein expression system (e.g., *E. coli* lysate) should be added 1:10 to blocking buffer used for dilution of sera and quality control antibodies in order to block any antibodies directed against these components. Slide configurations are available with a lower number of larger pads on each slide to accommodate a higher number of antigens per array. For this study, we used a GeneMachines OmniGrid 100 microarray printer that utilizes pins that directly contact the nitrocellulose surface to deposit spots on the array. While this microarray printer is no longer commercially available, other commercially available microarray printers can be used in this protocol but may require custom pins (either contact or non-contact) and software and should have sufficient spot resolution and compatibility with slides and imager. Depending on reactivity of sera, dilutions from 1:50 to 1:400 can be used. Serum-free buffer can be used as a negative control, while monoclonal antibodies known to bind to antigen can be used as a positive control.

Users should be aware of a few troubleshooting issues. The purpose of the quality control check using antibodies to the His tag is to check for any antigens for which printing was not successful. Any spots that consistently give low signal in quality control check of multiple arrays likely represent insufficient antigen printed. Possible reasons include aggregation or precipitation or antigen in source plate or poor contact between printing pin and microarray slide due to variable thickness of nitrocellulose pad. At this point, we do not perform assay normalization based on quantitative results of the quality control check, given that the detected binding of anti-His antibodies can be influenced by availability of this tag, which depends on the 3-dimensional conformation specific to each antigen.

The influenza antigen microarray provides several advantages over traditional methods such as ELISA and is complementary to functional assays such as HAI and MN. The 16-pad protein microarray is a sample-sparing technology with capacity to measure antibodies of multiple isotypes simultaneously against approximately 300 antigens from 1 µL of serum. The number of antigens can be increased to the thousands by decreasing the number of pads per slides. The multiplexed assay also spares personnel time and consumable resources, given that hundreds of sera can be probed for antibodies in 2 days, and all materials other than slides and reagents are re-usable so do not generate large amounts of plastic waste.

While microarray printers may not be widely distributed, microarray slides can be printed at a centralized location and then transported to the end user for probing. The only equipment required for probing is the low-cost and portable imager. The goal of disseminating this protocol is to make utilization of this technique more widespread.

The main limitations of the influenza antigen microarray are the inability to characterize the function and kinetics of the detected antibodies. The microarray is detecting a polyclonal set of binding antibodies for each antigen. These antibodies may or may not be functional in neutralizing virus in HAI and MN assays. However, HAI and MN assays require live virus culture with associated need for specialized facilities with high-level biosafety cabinets to test for antibodies against avian subtypes of influenza, whereas the protein microarray does not involve live virus components so can be utilized in any basic laboratory. With respect to binding kinetics, a single dilution of serum probed with an array containing a single concentration of each antigen yields a single data point, which represents a composite of quantity and affinity summated over all antibodies that bind to the antigen. To fully resolve the antigen-antibody binding kinetics, multiple antigen concentrations and/or serial dilutions of sera are required.

Despite these limitations, the influenza antigen microarray is a useful tool to characterize breadth of influenza antibodies across the antigenic landscape that can complement functional assays that are more limited in throughput and availability.

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The authors have no disclosures.

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Figure 1

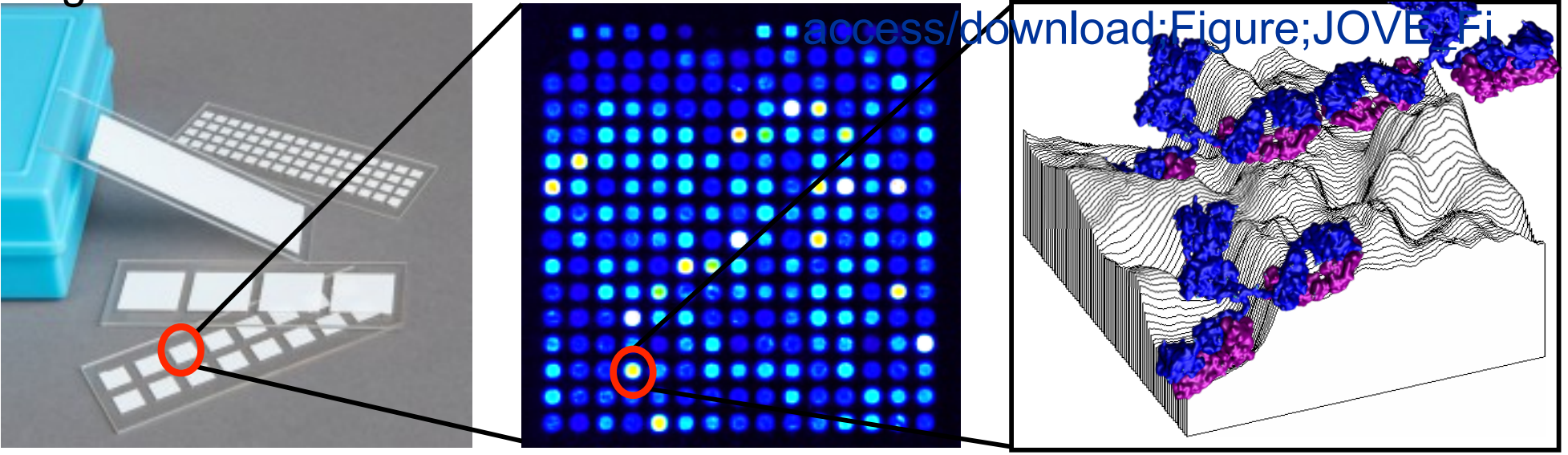


Figure 2

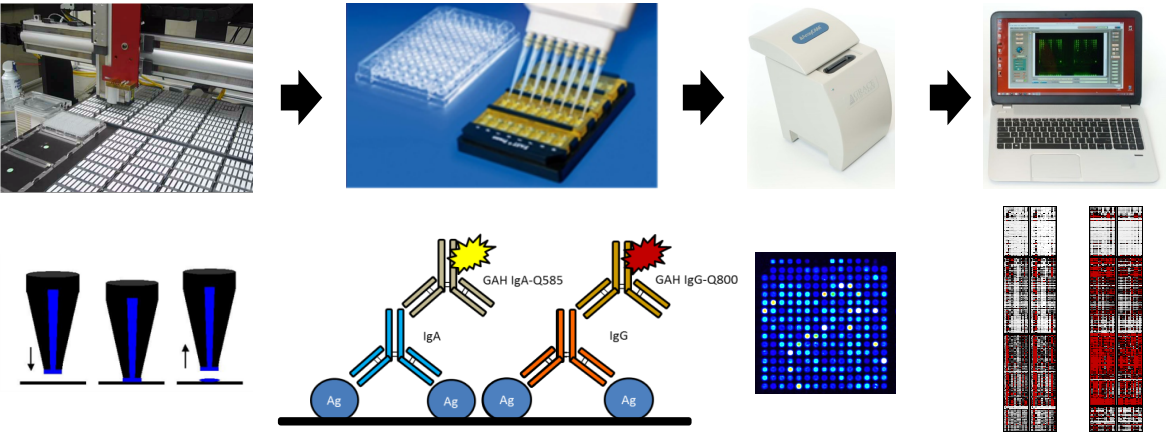


Figure 3

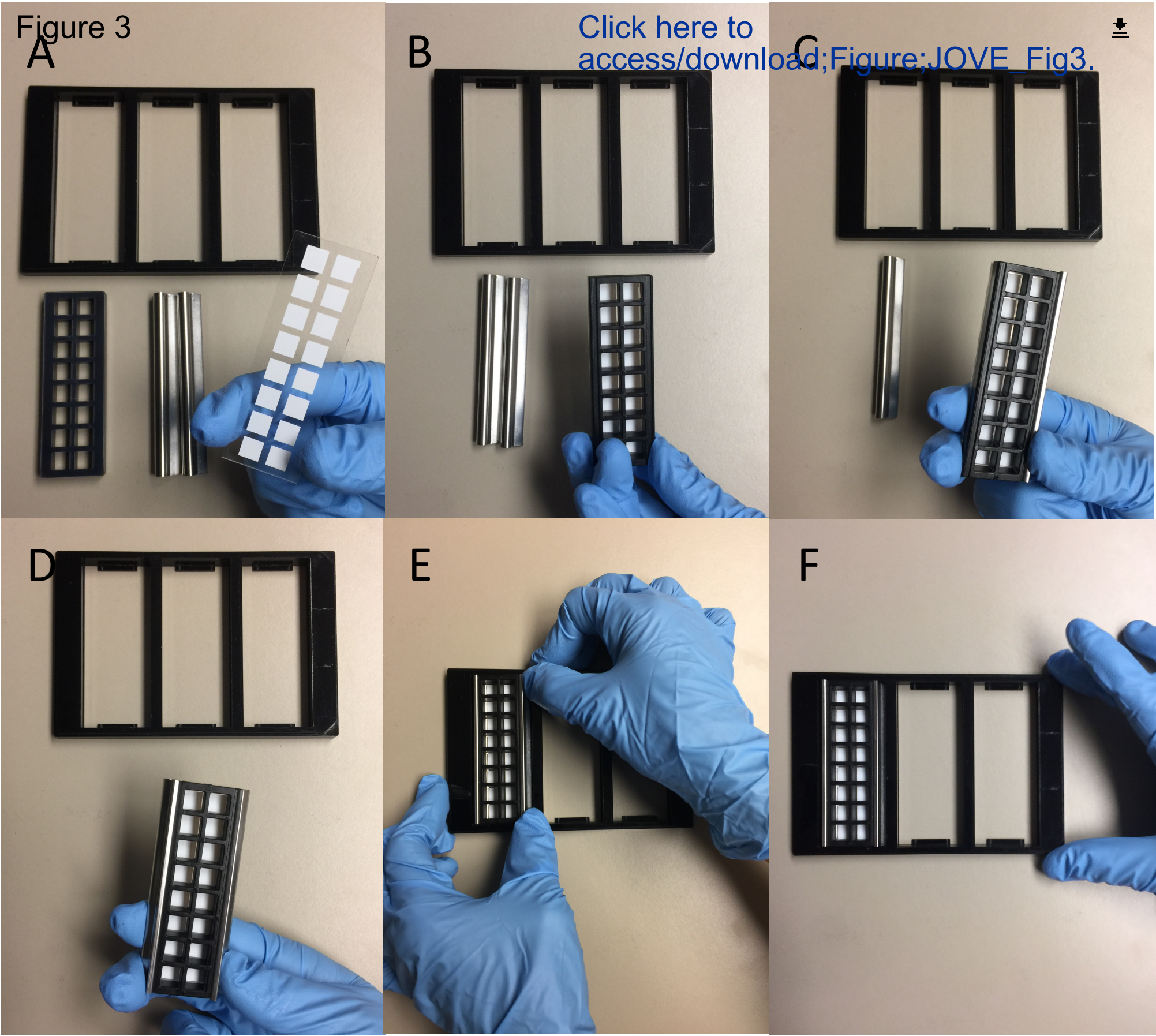


Figure 4

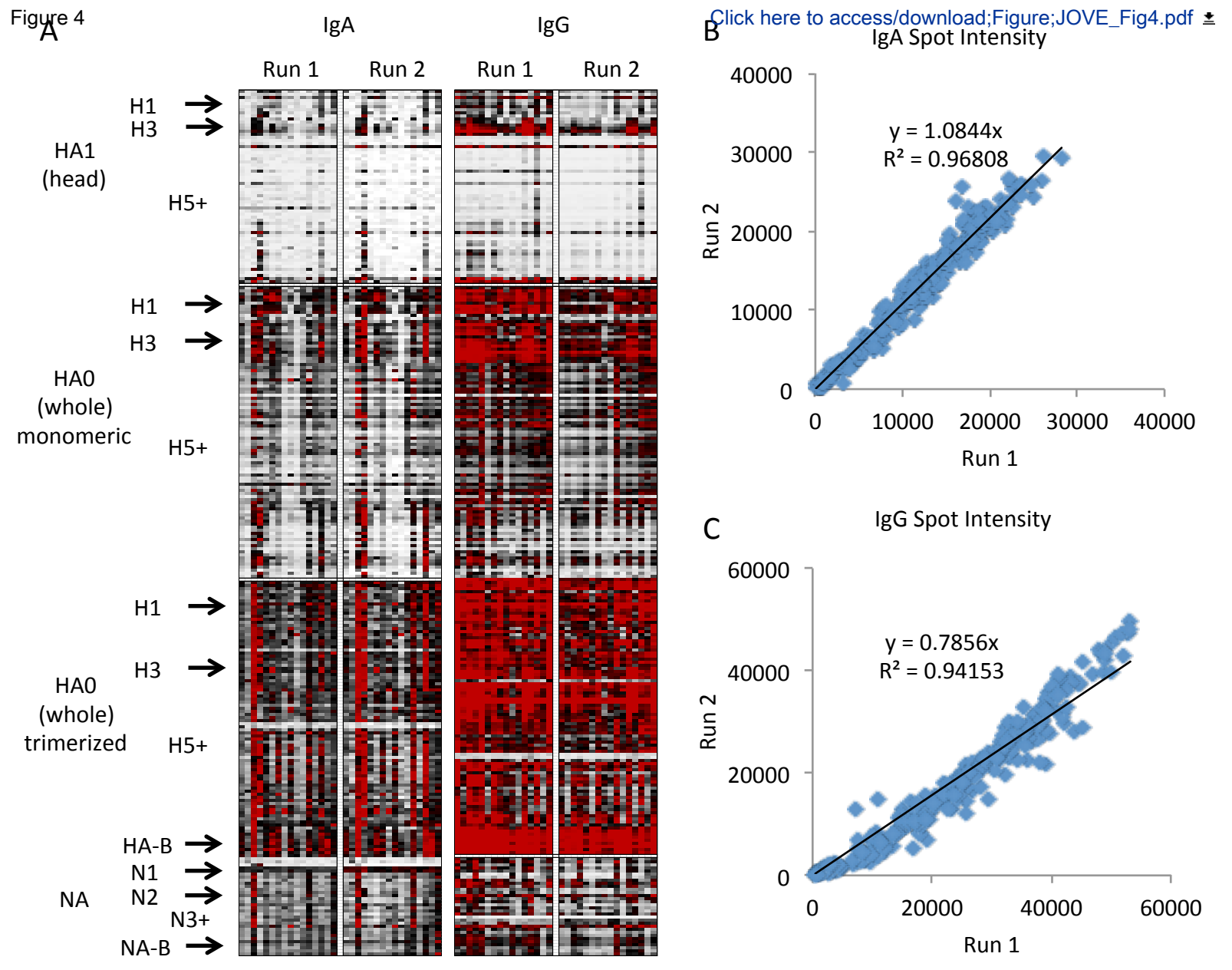
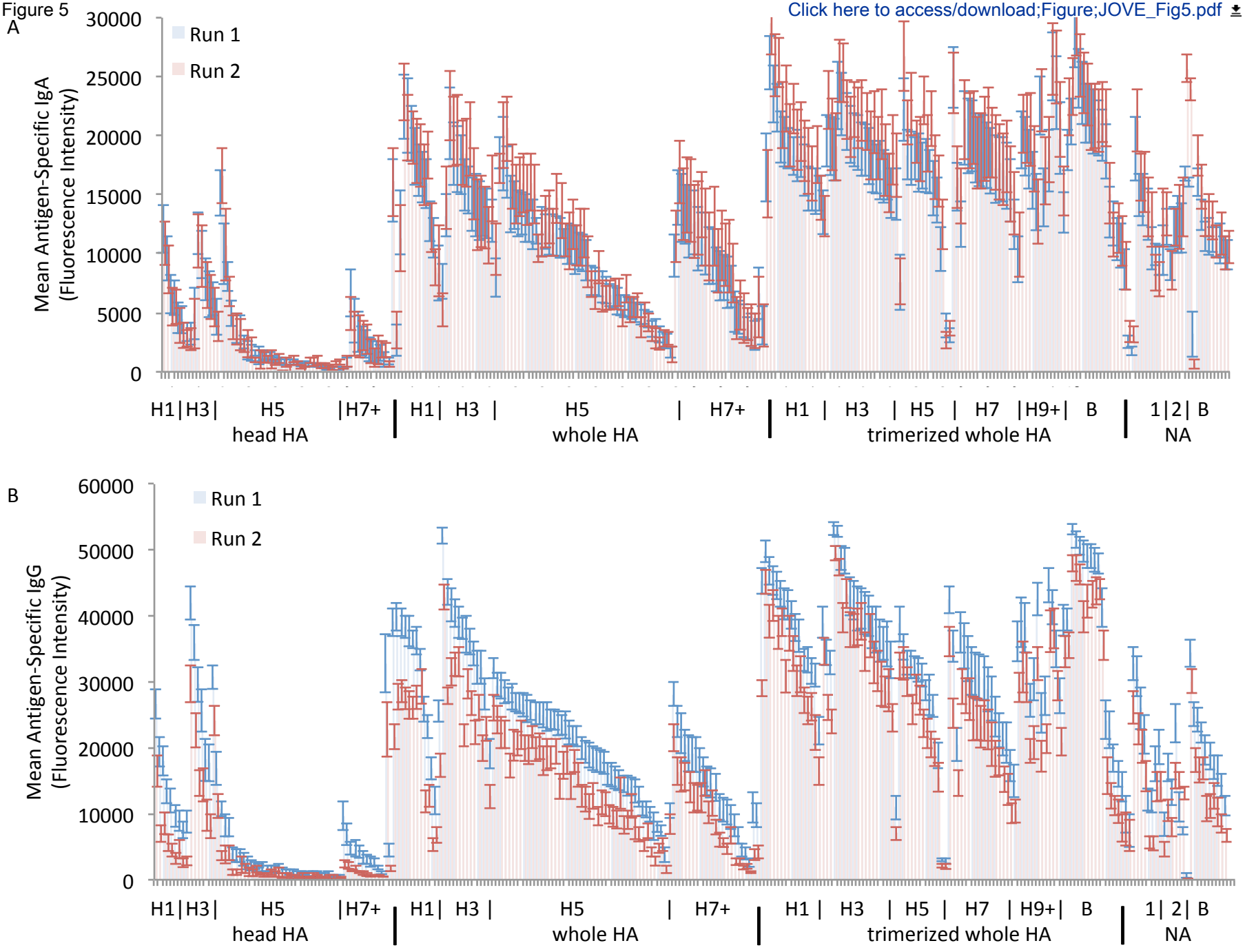


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
16-pad nitrocellulose-coated glass slides	Grace Bio Labs	305016	
1x GVS FAST blocking buffer	Fischer Scientific	10485356	
ArrayCam portable imager	Grace Bio Labs	400S	Other imaging devices can be used to visualize slides if capable of achieving the resolution of the microarray spots and the excitation and emission wavelengths of the quantum dots.
Biotin-conjugated goat anti-mouse-IgG antibody	Thermo Fischer	31800	
HiBase 384-well plate	Greiner Bio-One	T-3037-11	
Microarray pins	ArrayIt	GMP2	Each different microarray printer may require its own custom microarray pins.
Mouse monoclonal poly-His antibody	Sigma-Aldrich	H1029	
OmniGrid 100 microarray printer	GeneMachines		The version of the microarray printer used in this work is no longer commercially available, but the updated similar equipment is the OmniGrid Accent microarray printer from Digilab (Hopkinton, MA), and the same protocol can be carried out with most commercially available microarray printers.

ProPlate slide chambers	Grace Bio Labs	246890
ProPlate slide clips	Grace Bio Labs	204838
ProPlate slide frames	Grace Bio Labs	246879
Quantum dot 585 nm conjugated goat anti-human-IgA antibody	Grace Bio Labs	110620
Quantum dot 585 nm streptavidin conjugate	Thermo Fischer	Q10111MP
Quantum dot 800 nm conjugated goat anti-human-IgG antibody	Grace Bio Labs	110610

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Title of Article: Use of Influenza Antigen Microarray to Measure Breadth of Serum Antibodies Across Virus Subtypes

Author(s): Saahir Khan, Aarti Jain, Omid Taghavian, Rie Nakajima, Algis Jasinskas, Medalyn Supnet, Jiin Felgner, Jennifer Davies, Rafael R. de Assis, Sharon Jan, Joshua Obiero, Erwin Strahsburger, Egest J. Pone, Li Liang, Philip L. Felgner, D. Huw Davies

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April 29, 2019

Dr. Vineeta Bajaj
Review Editor
Journal of Visualized Experiments
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Cambridge, MA 02140

Dr. Bajaj:

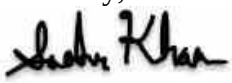
Please find enclosed our group's revised manuscript entitled "Use of Influenza Antigen Microarray to Measure Breadth of Serum Antibodies Across Virus Subtypes" for invited submission to the Journal of Visualized Experiments, Immunology and Infection Section. This manuscript describes a novel methodology to measure the breadth of serum antibodies to influenza using an antigen microarray. The data presented for demonstration of this methodology has not been published elsewhere.

The authors have no conflicts of interest to disclose.

We have attached below our response to the reviewers. We have also incorporated feedback from the editors. We would like to thank the reviewers and editors for helping us to improve our manuscript.

Please do not hesitate to contact us if we can provide any information that will be helpful in your review. Thank you for the opportunity to submit this manuscript.

Sincerely,



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Response to Reviewer 1:

Comment	Response
1) In order to place this work in the appropriate scholarly context, it is important to note that influenza antigen microarrays have been produced by several other groups. Key references include Mace et al, Talanta, 2011, 83, 1000; Koopmans et al., Clin. Microb. Infect. 2011, 18, 797; Bucukovski et al, PLoS ONE 2015, 10, e0134484; and Meade et al, Emerg. Microbes Infect. 2017, 6, e110.	We have included citations to these excellent examples of antecedent work in the revised introduction.
2) Page 2, line 131: "microarray printer" can mean several different possible technologies. From what's described here, the authors use a non contact, pin-based printer; this should be specified. This is particularly important here since the manufacturer the authors reference (Gene Machines) no longer exists (this is acknowledged in the table of materials, but it would be useful to have in the main text as well). The type of pin should also be described in addition to the catalog number.	We have clarified in the discussion the particulars of the microarray printer used in this study and noted that other commercially available microarray printers can be used to produce arrays although specific software or pins may vary.
3) ScanArray Express is (as far as this reviewer can determine) no longer available. What alternatives are available to those who want to replicate the method?	We have removed references to ScanArray Express and described in more detail how to analyze the data using the built-in software for the ArrayCam imager.
4) Figure 1 is described as "one antigen adsorbed onto the 3-dimensional topography of the nitrocellulose surface", but the image appears to be of an antibody.	We have modified the figure legend to clarify that what is shown is serum antibodies bound to antigen-coated nitrocellulose slides.

Response to Reviewer 2:

Comment	Response
The authors do a control based on the HIStag, to check whether each protein is printed (point 1.3 in the protocol). From the manuscript it doesn`t come clear whether they correct for any variations per antigen, slide, or slide batch differences. They also doesn't tell what came out of the HIStag experiment, and whether this is used as a quality control for the final result.	We have provided additional detail in the discussion about the role and limitations of the anti-His antibody quality control check, including reasons why normalization of assay results based on this quality control check is not routinely performed.
The next experiment done, to determine the level of binding IgA and IgG in human serum samples, was done twice and results are shown in figure 4 and 5. The IgA was very reproducible, but the IgG result shows differences, although the antibody binding profile for all antigens remains the same. The authors argue that this was caused by changing lot number of the anti IgG secondary antibody. However, this cannot be concluded from just two experiments. At least they should confirm it can be reproducible, when using the	We included this result to illustrate how to deal with small variations in microarray data expected between experimental runs. In practice, the large number of samples to be tested on a microarray sometimes requires different batches of secondary antibody to be used. We have included in the results additional clarification on how to correct for these anticipated issues by including shared samples between different experimental runs with different batches of antibodies.

same batch of secondary antibody.	
A lot of the protein microarray papers describe massive numbers of antigens printed on a single slide (or other format) and the result is presented as a whole. Biggest issue against those papers is lack of validation to most of the antigens presented on the protein microarray. Serological assays are known to have a certain amount of variability over time. When doing a single ELISA experiment, one would include a positive, negative and/or a blank control on each plate. When doing protein microarray you should also include controls to keep control on the test result. Can the authors explain how to keep control on the quality of your test result regarding all proteins included on the protein microarray and what controls should be used to know whether the result of a single experiment is valid?	We have included in the discussion the recommendation to use serum-free buffer as negative control for all antigens and monoclonal antibody as positive control for particular antigens. While monoclonal antibodies are unlikely to exist for all antigens on a microarray, and use of large numbers of monoclonal antibodies as positive controls may be prohibitive in terms of cost, this methodology can be used for a subset of antigens with known monoclonal antibodies.
According to the authors the protein "microarray is a useful tool to characterize breadth of influenza antibodies across the antigenic landscape". Outcome of the protein microarray in this manuscript is now shown as a whole and not very specific, which makes it difficult to interpret. Having a quick look at it, tells me that whole HA, whether it is a trimer or not, shows massive cross reaction. For the HA1 recombinant proteins, some of the H5 recombinant proteins show higher signals compared to H1 or H3. In humans, one would expect high responses to the influenza virus strains circulating in the human population, H1, H3 and influenza B, but not to H5. Based on the high cross-reactive responses the protein microarray described in this manuscript seems not very useful, in contrast to what the authors say. Can you give a better description of the result and how this protein microarray can be used to measure the breadth of the responses or can you refer to another article, as this manuscript is mainly focused on the description of the method, and, show more validation work done on the protein microarray (for example; reproducibility of intra (on spot level) and inter slide results). This kind of validation information would be more helpful in this paper.	We have included additional explanation as to why antibodies to the whole HA antigens of non-clinical subtypes show cross-reactivity (due to cross-reactive stem antibodies generated against conserved stem regions of clinical HA subtypes) and pointed out how this expected result illustrates the importance of including both head group and whole molecule forms of HA on the array.
Sentence 69-72; "In addition to the…. ...as occurred with the 2009 H1N1 Influenza A strain". So far there were luckily no artificially or bioterrorism worldwide pandemic, and, the 2009 H1N1 strain might have spread rapidly, but wasn't highly lethal. I would explain this differently.	We have removed the reference to the 2009 H1N1 epidemic in this sentence.
3. Figure 4 and 5; The reader can get an overview of	We have added clarification in the results section

the full response, but details cannot be seen from these figures. This can be made better by explaining what is depicted, for example; What does the plus sign mean in those figures? For example "H5+", does it mean H5 to H18, but where are H2 and H4? Please make the figures easier to understand and read. Does not account for figure 4B and 4C, those are good.	explaining the labeling of Figures 4 and 5, with only the clinically relevant and most represented subtypes explicitly labeled to save space, with other subtypes included within the ordering by number. We have also pointed the reader to Appendix A where detailed information on strains and subtypes included on the array is presented.

Array Index	Antigen Source	Antigen ID or Cat. No.	Molecular Form	Virus Subtype	Virus Strain
1	Control	Fiducial			
2	Control	PBST wash			
3	Control	PBST wash			
4	Control	PBST wash			
5	Control	PBST wash			
6	Control	Fiducial			
7	Control	PBST wash			
8	Control	PBST wash			
9	Control	PBST wash			
10	Control	PBST wash			
11	Control	PBST wash			
12	Control	Fiducial			
13	Control	PBST wash			
14	Control	PBST wash			
15	Control	PBST wash			
16	Control	PBST wash			
17	Control	PBST wash			
18	Control	Fiducial			
19	Control	PBST wash			
20	Control	IgGmix-0.3			
21	Control	IgGmix-0.1			
22	Control	PBST wash			
23	Control	a-Hu IgG 0.3			
24	Control	a-Hu IgG 0.1			
25	Control	Hu IgA_0.3			
26	Control	Hu IgA_0.1			
27	Control	PBST wash			
28	Control	a-Hu IgA_0.3			
29	Control	a-Hu IgA_0.1			
30	Control	PBST wash			
31	Sino	10003-V04H2	HA2	H5N1	A/VietNam/1203/2004
32	Sino	11052-V08H	HA0	H1N1	A/Brisbane/59/2007
33	Sino	11056-V08H	HA0	H3N2	A/Brisbane/10/2007 (H)
34	Sino	10003-V06H1	HA1	H5N1	A/VietNam/1203/2004
35	Sino	11052-V08H1	HA1	H1N1	A/Brisbane/59/2007
36	Sino	11056-V08H1	HA1	H3N2	A/Brisbane/10/2007
37	Sino	10003-V06H3	HA0	H5N1	A/VietNam/1203/2004
38	Sino	11055-V08B	HA0	H1N1	A/California/04/2009 (B)
39	Sino	11059-V08B1	HA0	H5N1	A/bar-headed goose/Qinghai/14/2008 (B)
40	Sino	11048-V06H1	HA0	H5N1	A/Anhui/1/2005
41	Sino	11055-V08H	HA0	H1N1	A/California/04/2009 (H)
42	Sino	11059-V08H1	HA0	H5N1	A/bar-headed goose/Qinghai/14/2008 (H)
43	Sino	11048-V08H1	HA0	H5N1	A/Anhui/1/2005 (H)
44	Sino	40015-V08B	HA0	H5N1	A/Hubei/1/2010 (B)
45	FKL	gyrfalcon H5N8	HA0	H5N8	gyrfalcon/Washington/41088-6/2014 (H5N8)
46	Sino	11061-V08H1	HA0	H5N1	A/turkey/Turkey/1/2005
47	Sino	40109-VNAHC	NA	H7N9	A/Shanghai/1/2013
48	FKL	gyrfalcon H5N8 batch#12	HA0	H5N8	gyrfalcon/Washington/41088-6/2014 (H5N8)
49	Sino	11048-V08B	HA0	H5N1	A/Anhui/1/2005 (B)
50	Sino	11055-V08H2	HA0	H1N1	A/California/04/2009
51	Sino	11059-V08H2	HA0	H5N1	A/bar-headed goose/Qinghai/14/2008
52	Sino	11048-V08H2	HA1	H5N1	A/Anhui/1/2005
53	Sino	11055-V08H4	HA1	H1N1	A/California/04/2009
54	Sino	11060-V08H1	HA0	H5N1	A/Indonesia/5/2005
55	Sino	11048-V08H4	HA0	H5N1	A/Anhui/1/2005
56	Sino	11055-VNAB	HA0	H1N1	A/California/04/2009
57	Sino	11060-V08H2	HA0	H5N1	A/Indonesia/5/2005

58	Sino	11048-VNAH2	HA1	H5N1	A/Anhui/1/2005
59	Sino	11056-V08B	HA0	H3N2	A/Brisbane/10/2007 (B)
60	Sino	11061-V08H2	HA0	H5N1	A/turkey/Turkey/1/2005
61	Sino	11062-V08H1	HA0	H5N1	A/Vietnam/1194/2004
62	Sino	11229-V08H	HA0	H9N2	A/Hong Kong/1073/99
63	Sino	11687-V08H1	HA1	H1N1	A/Ohio/UR06-0091/2007
64	Sino	11062-V08H2	HA0	H5N1	A/Vietnam/1194/2004
65	Sino	11229-V08H1	HA1	H9N2	A/HongKong/1073/99
66	Sino	11689-V08H	HA0	H5N1	A/Hong Kong/483/97
67	Sino	11068-V08H	HA0	H1N1	A/Brevig Mission/1/1918
68	Sino	11683-V08H	HA0	H1N1	A/New Caledonia/20/99
69	Sino	11689-V08H1	HA1	H5N1	A/Hong Kong/483/97
70	Sino	11068-V08H1	HA1	H1N1	A/Brevig Mission/1/1918
71	Sino	11683-V08H1	HA1	H1N1	A/New Caledonia/20/99
72	Sino	11690-V08H	HA0	H5N1	A/goose/Guiyang/337/2006
73	Sino	11082-V08B	HA0	H7N7	A/Netherlands/219/03
74	Sino	11685-V08H	HA0	H1N3	A/duck/NZL/160/1976
75	Sino	11690-V08H1	HA1	H5N1	A/goose/Guiyang/337/2006
76	Sino	11082-V08H1	HA1	H7N7	A/Netherlands/219/03
77	Sino	11685-V08H1	HA1	H1N3	A/duck/NZL/160/1976
78	Sino	11693-V08H	HA0	H10N3	A/duck/Hong Kong/786/1979 (H)
79	Sino	11212-V08B	HA0	H7N7	A/chicken/Netherlands/1/03
80	Sino	11686-V08H1	HA1	H5N1	A/chicken/Egypt/2253-1/2006
81	Sino	11694-V08H	HA0	H5N1	A/Japanese white-eye/Hong Kong/1038/2006
82	Sino	11212-V08H1	HA1	H7N7	A/chicken/Netherlands/1/03
83	Sino	11687-V08H	HA0	H1N1	A/Ohio/UR06-0091/2007
84	Sino	11694-V08H1	HA1	H5N1	A/Japanese white-eye/Hong Kong/1038/2006
85	Sino	11696-V08H	HA0	H5N3	A/duck/Hokkaido/167/2007
86	Sino	11700-V08H	HA0	H5N1	A/Common magpie/Hong Kong/2256/2006
87	Sino	11709-V08H1	HA1	H5N1	A/whooper swan/Mongolia/244/2005
88	Sino	11696-V08H1	HA1	H5N3	A/duck/Hokkaido/167/2007
89	Sino	11700-V08H1	HA1	H5N1	A/Common magpie/Hong Kong/2256/2006
90	Sino	11710-V08B	HA0	H5N1	A/Cambodia/R0405050/2007 (B)
91	Sino	11697-V08H	HA0	H5N1	A/Egypt/2321-NAMRU3/2007
92	Sino	11701-V08H1	HA1	H5N1	A/duck/Laos/3295/2006
93	Sino	11710-V08H	HA0	H5N1	A/Cambodia/R0405050/2007 (H)
94	Sino	11697-V08H1	HA1	H5N1	A/Egypt/2321-NAMRU3/2007
95	Sino	11702-V08H	HA0	H5N1	A/Egypt/N05056/2009
96	Sino	11710-V08H1	HA1	H5N1	A/Cambodia/R0405050/2007
97	Sino	11698-V08H	HA0	H5N1	A/duck/Hunan/795/2002
98	Sino	11702-V08H1	HA1	H5N1	A/Egypt/N05056/2009
99	Sino	11712-V08B	HA0	H5N1	A/chicken/India/NIV33487/06 (B)
100	Sino	11698-V08H1	HA1	H5N1	A/duck/Hunan/795/2002
101	Sino	11708-V08H	HA0	H1N1	A/Solomon Islands/3/2006
102	Sino	11712-V08H	HA0	H5N1	A/chicken/India/NIV33487/06 (H)
103	Sino	11699-V08H	HA0	H5N2	A/American green-winged teal/California/HKWF609/2007
104	Sino	11708-V08H1	HA1	H1N1	A/Solomon Islands/3/2006
105	Sino	11712-V08H1	HA1	H5N1	A/chicken/India/NIV33487/06
106	Sino	11699-V08H1	HA1	H5N2	A/American green-winged teal/California/HKWF609/2007
107	Sino	11709-V08H	HA0	H5N1	A/whooper swan/Mongolia/244/2005
108	Sino	11713-V08H	HA0	H5N1	A/Hong kong/213/2003
109	Sino	11713-V08H1	HA1	H5N1	A/Hong kong/213/2003
110	Sino	11972-V08H	HA0	H3N2	A/Wisconsin/67/X-161/2005 (H)
111	Sino	40022-V08H1	HA1	H5N1	A/Vietnam/UT31413II/2008
112	Sino	11715-V08H	HA0	H3N2	A/Wyoming/03/2003
113	Sino	11972-V08H1	HA1	H3N2	A/Wisconsin/67/X-161/2005
114	Sino	40024-V08B	HA0	H5N1	A/Goose/Guangdong/1/96
115	Sino	11715-V08H1	HA1	H3N2	A/Wyoming/03/2003

116	Sino	40001-V08H	HA0	H5N1	A/Duck/Hong Kong/p46/97
117	Sino	40043-V08H	HA0	H3N2	A/Perth/16/2009
118	Sino	11716-V08H	HA0	B	B/Malaysia/2506/2004
119	Sino	40001-V08H1	HA1	H5N1	A/Duck/Hong Kong/p46/97
120	Sino	40043-V08H1	HA1	H3N2	A/Perth/16/2009
121	Sino	11716-V08H1	HA1	B	B/Malaysia/2506/2004
122	Sino	40004-V08H	HA0	H5N1	A/Xinjiang/1/2006
123	Sino	40044-V08H	HA0	H5N1	A/common magpie/Hong Kong/5052/2007
124	Sino	11717-V08H	HA0	H5N8	A/duck/NY/191255-59/2002
125	Sino	40004-V08H1	HA1	H5N1	A/Xinjiang/1/2006
126	Sino	40044-V08H1	HA1	H5N1	A/common magpie/Hong Kong/5052/2007
127	Sino	11717-V08H1	HA1	H5N8	A/duck/NY/191255-59/2002
128	Sino	40014-V08H1	HA1	H5N2	A/ostrich/South Africa/AI1091/2006
129	Sino	40049-V08H1	HA1	H5N1	A/Egypt/3300-NAMRU3/2008
130	Sino	11972-V08B	HA0	H3N2	A/Wisconsin/67/X-161/2005 (B)
131	Sino	40015-V08H	HA0	H5N1	A/Hubei/1/2010 (H)
132	Sino	40060-V08H1	HA1	H5N1	A/Hubei/1/2010
133	Sino	40064-V07H	NA	H5N1	A/Thailand/1(KAN-1)/2004
134	Sino	40104-V08H	HA0	H7N9	A/Shanghai/1/2013 (Hi)
135	Sino	40111-V08B	NP	H7N9	A/Shanghai/2/2013
136	Sino	40064-V07H-B	NA	H5N1	A/Thailand/1(KAN-1)/2004
137	Sino	40104-V08H1	HA1	H7N9	A/Shanghai/1/2013 (H)
138	Sino	40116-V08B	HA0	H3N2	A/Hong Kong/1/1968
139	Sino	40065-V08H1	HA1	H5N1	A/Thailand/1(KAN-1)/2004
140	Sino	40104-V08H4	HA0	H7N9	A/Shanghai/1/2013 (Hii)
141	Sino	40116-V08H1	HA1	H3N2	A/Hong Kong/1/1968
142	Sino	40103-V08H	HA0	H7N9	A/Anhui/1/2013 (H)
143	Sino	40105-V08B	HA0	H7N9	A/Hangzhou/1/2013 (B)
144	Sino	40117-V08B	HA0	H5N1	A/bar-headed goose/Qinghai/1A/2005
145	Sino	40103-V08H1	HA1	H7N9	A/Anhui/1/2013
146	Sino	40105-V08H	HA0	H7N9	A/Hangzhou/1/2013 (H)
147	Sino	40117-V08H1	HA1	H5N1	A/bar-headed goose/Qinghai/1A/2005
148	Sino	40103-V08H4	HA0	H7N9	A/Anhui/1/2013
149	Sino	40105-V08H1	HA1	H7N9	A/Hangzhou/1/2013
150	Sino	40119-V08B	HA0	H2N2	A/Guiyang/1/1957
151	Sino	40104-V08B	HA0	H7N9	A/Shanghai/1/2013 (B)
152	Sino	40106-V08H	HA0	H7N9	A/Pigeon/Shanghai/S1069/2013 (H)
153	Sino	40119-V08H1	HA1	H2N2	A/Guiyang/1/1957
154	Sino	40104-V08B1	HA1	H7N9	A/Shanghai/1/2013 (B)
155	Sino	40109-V07H	NA	H7N9	A/Shanghai/1/2013
156	Sino	40120-V08B	HA0	H3N2	A/Fujian/411/2002
157	Sino	40128-V08B	HA0	H7N3	A/turkey/Italy/214845/2002
158	Sino	40154-V08B	HA0	H3N2	A/Moscow/10/1999
159	Sino	40164-V08H1	HA1	H5N8	A/turkey/Ireland/1378/1983
160	Sino	40126-V08B	HA0	H7N9	A/Shanghai/4664T/2013
161	Sino	40153-V08B	HA0	H3N2	A/Babol/36/2005
162	Sino	40164-V08B2	HA2	H5N8	A/turkey/Ireland/1378/1983
163	Sino	40125-V08B	HA0	H7N9	A/Zhejiang/1/2013
164	Sino	40149-V08B	HA0	H3N2	A/Sydney/5/1997
165	Sino	40160-V08H1	HA1	H5N1	A/barn swallow/Hong Kong/D10-1161/2010
166	Sino	40123-V08B	HA0	H7N9	A/Hangzhou/3/2013
167	Sino	40146-V08B	HA0	H3N2	A/Hong Kong/CUHK31987/2011
168	Sino	40160-V08B1	HA0	H5N1	A/barnswallow/HongKong/D10-1161/2010 (B)
169	Sino	40128-V08H1	HA1	H7N3	A/turkey/Italy/214845/2002
170	Sino	40158-V08B	HA0	H5N1	A/chicken/VietNam/NCVD-016/2008 (B)
171	Sino	40165-V08B	HA0	H5N9	A/chicken/Italy/22A/1998
172	Sino	40129-V08H1	HA1	H7N3	A/chicken/SK/HR-00011/2007
173	Sino	40158-V08B2	HA0	H5N1	A/chicken/VietNam/NCVD-016/2008 (B)

174	Sino	40165-V08H1	HA1	H5N9	A/chicken/Italy/22A/1998
175	Sino	40134-V08B	HA0	H1N1	A/USSR/90/1977
176	Sino	40158-V08H1	HA1	H5N1	A/chicken/VietNam/NCVD-016/2008
177	Sino	40168-V08B	HA0	H6N8	A/mallard/Ohio/217/1998
178	Sino	40134-V08H1	HA1	H1N1	A/USSR/90/1977
179	Sino	40160-V08B	HA0	H5N1	A/barnswallow/HongKong/D10-1161/2010 (B)
180	Sino	40168-V08H1	HA1	H6N8	A/mallard/Ohio/217/1998
181	Sino	40169-V08H1	HA1	H7N1	A/turkey/Italy/4602/99
182	Sino	40325-V08B	HA0	H7N9	A/Zhejiang/DTID-ZJU10/2013 (B)
183	Sino	40172-V08B	HA0	H7N8	A/mallard/Netherlands/33/2006
184	Sino	40170-V08B	HA0	H7N2	A/ruddy turnstone/New Jersey/563/2006
185	Sino	40325-V08H	HA0	H7N9	A/Zhejiang/DTID-ZJU10/2013 (H)
186	Sino	40172-V08H1	HA1	H7N8	A/mallard/Netherlands/33/2006
187	Sino	40170-V08H1	HA1	H7N2	A/ruddy turnstone/New Jersey/563/2006
188	Sino	40354-V08B	HA0	H3N2	A/Texas/50/2012
189	Sino	40239-V08B	HA0	H7N9	A/Shanghai/2/2013 (B)
190	Sino	40171-V08B	HA0	H7N7	A/equine/Kentucky/1a/1975
191	Sino	40354-V08H1	HA1	H3N2	A/Texas/50/2012
192	Sino	40239-V08H	HA0	H7N9	A/Shanghai/2/2013 (H)
193	Sino	40372-V08B	HA0	H5N1	A/chicken/Jilin/9/2004
194	Sino	11048-V08H1	HA0	H5N1	A/Anhui/1/2005 (H)
195	Sino	40015-V08B	HA0	H5N1	A/Hubei/1/2010 (B)
196	Sino	40372-V08H1	HA1	H5N1	A/chicken/Jilin/9/2004
197	Sino	11061-V08H1	HA0	H5N1	A/turkey/Turkey/1/2005
198	Sino	40109-VNAHC	NA	H7N9	A/Shanghai/1/2013
199	FKL	Guan H7	HA0	H7N9	A/Guangdong/17SF003/2016
200	FKL	Hunan H7	HA0	H7N9	A/Hunan/02285/2017
201	FKL	Vn04 H5 (HA)	HA0	H5N1	A/Vietnam/1204/2004 (H5)
202	FKL	SHT	HA0	H7N9	A/Shanghai/1/2013 (H7)
203	FKL	Indo H5	HA0	H5N1	A/Indonesia/05/2005 (H5)
204	FKL	Vn04 NA	NA	H5N1	A/Vietnam/1204/2004 (N1)
205	FKL	Perth H3	HA0	H3N2	A/Perth/16/2009 (H3)
206	FKL	B/Yam HA	HA0	B	B/Yamagata/16/1988
207	FKL	Panama H3	HA0	H3N2	A/Panama/2007/1999 (H3)
208	FKL	H18	HA0	H18N11	A/bat/Peru/33/2010 (H18)
209	FKL	Cal09 HA	HA0	H1N1	A/California/04/2009 (H1)
210	FKL	Alabama H3	HA0	H3N2	A/Alabama/1/1981 (H3)
211	FKL	Pintail H5	HA0	H5N2	A/Northern Pintail/WA/40964/2014 (H5 from novel H5N2)
212	FKL	Wyo H3	HA0	H3N2	A/Wyoming/3/2003 (H3)
213	FKL	H2 Mal	HA0	H2N9	A/mallard/Netherlands/5/1999 (H2)
214	FKL	PR8 HA	HA0	H1N1	A/PR/8/1934 (H1)
215	FKL	B/Wisc HA	HA0	B	B/Wisconsin/1/2010
216	FKL	H13	HA0	H13	A/black headed gull/Sweden/1/1999 (H13)
217	FKL	Shenzen H5	HA0	H5N6	A/Shenzen/1/16 (H5 from lethal human H5N6 case)
218	FKL	JDH10	HA0	H10N8	A/Jiangxi/Donghu/346/2013 (H10)
219	FKL	1918	HA0	H1N1	A/South Carolina/1/1918 (H1)
220	FKL	dH3	HA0	H3	A/canine/Texas/12/2004 (H3 equine lineage-dog isolate)
221	FKL	SH3	HA0	H3N8	A/harbor seal/Massachusetts/1/2011 (H3)
222	FKL	WSN	HA0	H1N1	A/William Smith Neurotropic/1933 (H1)
223	FKL	H8	HA0	H8	A/mallard/Sweden/24/2002 (H8)
224	FKL	B/Mal HA	HA0	B	B/Malaysia/2506/2004
225	FKL	asH1	HA0	H1N1	A/swine/Jiangsu/40/2011
226	FKL	H6	HA0	H6	A/mallard/Sweden/81/2002 (H6)
227	FKL	BC04	HA0	H7N3	A/chicken/BC/CN-6/2004 (H7)
228	FKL	B/Phuket/HA	HA0	B	B/Phuket/3073/2013
229	FKL	Swiss H3	HA0	H3N2	A/Switzerland/9715293/2013
230	FKL	ckNL H5	NA	H17N10	A/chicken/Netherlands/14015531/2014 (N8 from novel H5N8)
231	FKL	H17	HA0	H17N10	A/yellow shouldered bat/Guatemala/06/2010 (H17)

232	FKL	H12	HA0	H12	A/mallard/Interior Alaska/7MP0167/2007 (H12)
233	FKL	H9 head	HA1	H9	H9 head-only
234	FKL	Phil82 H3	HA0	H3N2	A/Philippines/2/1982 (H3)
235	FKL	B/Vic HA	HA0	B	B/Victoria/2/1987
236	FKL	H11	HA0	H11	A/shoveler/Netherlands/18/1999 (H11)
237	FKL	B/Phu HA	HA0	B	B/Phuket/3073/2013
238	FKL	cH9/1 PR8	HA0	H9	cH9/1
239	FKL	AHT	HA0	H7N9	A/Anhui/1/2013 (H7)
240	FKL	DR13	HA0	H1N1	A/DR/7293/2013 (H1)
241	FKL	B/Lee HA	HA0	B	B/Lee/1940
242	FKL	B/Mass HA	HA0	B	B/Massachusetts/2/2012
243	FKL	ddH3	HA0	H3	A/canine/NY/120106.2/2011 (H3 equine lineage-dog isolate)
244	FKL	Mich15	HA0	H1N1	A/Michigan/45/15 (pdmH1N1)
245	FKL	H3v_05/15/2015	HA0	H3N2	A/Indiana/10/2011 (H3v)
246	FKL	gfH9 (sfH9)	HA0	H9N2	A/guinea fowl/Hong Kong/WF10/1999 (H9)
247	FKL	B/Flo HA	HA0	B	B/Florida/4/2006
248	FKL	H3v_09/17/2015	HA0	H3N2	A/Indiana/10/2011 (H3v)
249	FKL	ckH9 (c6H9)	HA0	H9N2	A/chicken/Hong Kong/G9/1997 (H9)
250	FKL	H4	HA0	H4N6	A/duck/Czech/1956 (H4)
251	FKL	Wisc H3	HA0	H3N2	A/Wisconsin/67/2005 (H3)
252	FKL	NC99 HA	HA0	H1N1	A/New Caledonia/20/1999 (H1)
253	FKL	HK17 H7	HA0	H7N9	A/HongKong/2014/2017
254	FKL	cH6/1 PR8 (6/IPR8)	HA0	H6	cH6/1
255	FKL	FM1	HA0	H1N1	A/Fort/Monmouth/1/1947 (H1)
256	FKL	NYC H7	HA0	H7N2	A/feline/New York/16-040082-1/2016
257	FKL	Chick it h7	HA0	H7	A/chicken/Italy/13474/1999 (H7)
258	FKL	Vic11 H3	HA0	H3N2	A/Victoria/361/2011 (H3)
259	FKL	Phil82 (H3)	HA0	H3N2	A/Philippines/2/1982 (H3)
260	FKL	H7 Mallard	HA0	H7N3	A/mallard/Netherlands/12/2000 (H7)
261	FKL	Swiss Miss H2	HA0	H2N3	A/swine/Missouri/4296424/2006 (H2)
262	FKL	H14_10/13/2014	HA0	H14	A/mallard/Gurjev/263/1982 (H14)
263	FKL	Denv h1	HA0	H1N1	A/Denver/1/1957 (H1)
264	FKL	Tx 91 h1	HA0	H1N1	A/Texas/36/1991 (H1)
265	FKL	H14_2/1/2017	HA0	H14	A/mallard/Gurjev/263/1982 (H14)
266	FKL	jal	HA0	H7N3	A/chicken/Jalisco/12283/2012 (H7)
267	FKL	TW H6	HA0	H6N1	A/Taiwan/2/13 (H6)
268	FKL	H15	HA0	H15	A/shearwater/West Australia/2576/1979 (H15)
269	FKL	Rhea H7	HA0	H7N1	A/rhea/North Carolina/39482/93 (H7)
270	FKL	USSR	HA0	H1N1	A/USSR/1977 (H1)
271	FKL	HK68 H3_6/6/2016	HA0	H3N2	A/Hong Kong/1/1968 (H3)
272	FKL	Jap57 H2	HA0	H2N2	A/Japan/305/1957 (H2)
273	FKL	H16	HA0	H16	A/black headed gull/Sweden/5/1999 (H16)
274	FKL	HK68 H3_08/21/2017	HA0	H3N2	A/Hong Kong/1/1968 (H3)
275	FKL	FM1	HA0	H1N1	A/Fort/Monmouth/1/1947 (H1)
276	FKL	B/Bris NA	NA	B	B/Brisbane/60/2008 (B-NA)
277	FKL	B/Flo NA	NA	B	B/Florida/4/2006 (B-NA)
278	FKL	N4	NA	N5	A/mallard/Sweden/24/2002 (N4)
279	FKL	B/Mal NA_2/2/2018	NA	B	B/Malaysia/2506/2004 (B-NA)
280	FKL	ckNL N8	HA0	H5N8	A/chicken/Netherlands/14015531/2014 (H5 from novel H5N8)
281	FKL	NC99 NA	NA	H1N1	A/New Caledonia/20/1999 (N1)
282	FKL	B/Mal NA_01/25/2018	NA	B	B/Malaysia/2506/2004 (B-NA)
283	FKL	PR8 NA	NA	H1N1	A/PR/8/1934 (N1)
284	FKL	Cal09 NA	NA	H1N1	A/California/04/2009 (N1)
285	FKL	HK14 N2	NA	H7N2	A/HongKong/2014/2017
286	FKL	B/Mal NA_09/25/2017	NA	B	B/Malaysia/2506/2004 (B-NA)
287	FKL	Hk68 NA	NA	H3N2	A/Hong Kong/1/1968 (N2)
288	FKL	HK14N2	NA	H7N2	A/HongKong/2014/2017
289	FKL	N7	NA	H5N8	A/mallard/IA/10BM01929/2010 (N7)

290	FKL	USSR	HA0	H1N1	A/USSR/1977 (H1)
291	FKL	B/Flo NA	NA	B	B/Florida/4/2006 (B-NA)
292	FKL	Pan99 NA	NA	H3N2	A/Panama/2007/1999 (N2)
293	FKL	Vic H3	HA0	H3N2	A/Victoria/361/2011 (H3)
294	FKL	TX12N2	NA	H9N2	A/Texas/50/2012 (N2)
295	FKL	SGN2	NA	H3N2	A/Singapore/1/1957 (N2)
296	FKL	N5	NA	N6	A/mallard/Sweden/86/2003 (N5)
297	FKL	B/ Wisc NA	NA	B	B/Wisconsin/1/2010 (B-NA)
298	FKL	N3	NA	N4	A/swine/Missouri/4296424/2006 (N3)
299	FKL	B/Yam	NA	B	B/Yamagata/16/1988 (B-NA)
300	FKL	N6	NA	N7	A/mallard/Netherlands/1/1999 (N6)
301	FKL	ckN2	NA	H2N3	A/chicken/Hong Kong/G9/1997 (N2)
302	FKL	Guan H7	HA0	H7N9	A/Guangdong/17SF003/2016
303	FKL	Hunan H7	HA0	H7N9	A/Hunan/02285/2017
304	FKL	B/Yam NA	NA	B	B/Yamagata/16/1988 (B-NA)
305	FKL	SHT	HA0	H7N9	A/Shanghai/1/2013 (H7)
306	FKL	Indo H5	HA0	H5N1	A/Indonesia/05/2005 (H5)
307	FKL	Vn04 H5 (HA)	HA0	H5N1	A/Vietnam/1204/2004 (H5)
308	FKL	gyrfalcon H5N8	HA0	H5N8	gyrfalcon/Washington/41088-6/2014 (H5N8)
309	FKL	gyrfalcon H5N8 batch#12	HA0	H5N8	gyrfalcon/Washington/41088-6/2014 (H5N8)
310	FKL	Vn04 NA	NA	H5N1	A/Vietnam/1204/2004 (N1)
311	FKL	Cal 09 H1	HA0	H1N1	A/California/04/2009 (H1)
312	FKL	Anhui07	HA0	H7N9	A/Anhui/1/2013 (H7)
313	FKL	Vn04H5	HA0	H5N1	A/Vietnam/1204/2004 (H5)
314	Control	Dye			
315	Control	Blank			
316	Control	Blank			
317	Control	Blank			
318	Control	Blank			
319	Control	Blank			
320	Control	Blank			
321	Control	Blank			
322	Control	Blank			
323	Control	Blank			
324	Control	Blank			