VaxArray for hemagglutinin and neuraminidase potency testing of influenza vaccines

Laura R. Kuck [a], Rose Byrne-Nash [a], Jacob Gillis [a], Katie Bueter [a], Laura K. Couzens [b], Maryna C. Eichelberger [b], Kathy L. Rowlen [a,*]

[a] InDevR Inc., 2100 Central Ave., Suite 106, Boulder, CO 80301, United States
[b] Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD 20993, United States

ARTICLE INFO

ABSTRACT

Practical methods to measure the potency of influenza vaccines are needed as alternatives for the standard single radial immunodiffusion (SRID) assay. VaxArray assays for influenza hemagglutinin (HA) and neuraminidase (NA) have been developed to address this need. In this report, we evaluate the use of these assays to assess the potency of HA and NA of an A/H3N2 subunit vaccine by determining the correlation between the amounts measured by VaxArray and the immunogenicity in mice. The antibody response after one and two doses of five formulations of the vaccine ranging from 5 µg/mL to 80 µg/mL of HA, was measured by hemagglutination inhibition (HAI) and neuraminidase inhibition (NAI) assays. For hemagglutinin, vaccine potency determined by VaxArray was equivalent to potency measured SRID and these amounts were predictive of immunogenicity, with excellent correlation between potency measured by VaxArray and the HAI geometric mean titers (GMT). Likewise, the amount of NA measured by VaxArray was predictive of the NAI GMT. The VaxArray NA assay reported non-detectable levels of intact NA for a sample that had been heat degraded at 56 °C for 20 h, demonstrating that the assay measures the native, active form of NA. Similarly, the HA potency measured by VaxArray in this heat-treated sample was very low when a monoclonal antibody was used to detect the amount of antigen bound. Importantly, the force degraded sample induced low HAI titers and the NAI titers were not measurable, supporting the conclusion that the VaxArray HA and NA assays measure the immunogenic forms of these A/H3N2 antigens. This study indicates that VaxArray assays can be used to assess the potency of HA and NA components in influenza vaccines as a proxy for immunogenicity.

1. Introduction

As highlighted in recent publications, the single radial immunodiffusion (SRID) assay has served the influenza vaccine industry well for forty years [1–3]. However, these sources also point out a number of limitations for SRID including the lengthy time required to develop reference reagents, inadequate sensitivity for dose-sparing vaccines, and its unsuitability for new influenza vaccines based on emerging platform technologies such as cell culture generated recombinant proteins or virus like particles produced in cell culture or plants [1–7]. Furthermore, there is a strong motivation in both public health and the flu vaccine industry to improve the efficacy of flu vaccines. One aspect of that drive is an effort to deepen our understanding of the role played by other viral proteins

in the vaccine, such as neuraminidase (NA), nucleoprotein (NP), and the matrix protein [8–12]. For example, recently a new focus group (NAction!) was created to promote research on the role of NA plays in flu vaccine efficacy [13]. Current regulations for lot release testing of influenza vaccines specify that the presence of NA must be confirmed, but there is no requirement for quantification and the levels of NA can vary significantly from one season to another [13–15].

To address the limitations of SRID and to enable rapid, quantitative assessment of neuraminidase in flu vaccines, we developed and previously reported on the VaxArray vaccine potency testing platform [16,17]. The system is based on a multiplexed immunoassay printed in a microarray format. For the "seasonal hemagglutinin" microarray, each array contains subtype specific monoclonal antibodies against all of the hemagglutinin (HA) antigens within quadrivalent seasonal vaccines [16]. Previous studies demonstrated good accuracy, precision, applicability to dose-sparing vaccines due to excellent sensitivity, and applicability to

---

* Corresponding author.
  E-mail address: rowlen@indevr.com (K.L. Rowlen).

Table 1
Standards and samples included in the study.

Reference antigen	Lot # or ID	Source	[HA] by SRID (µg/mL)	[HA] by paBCA (µg/mL)	[NA] by IDMS (µg/mL)
Standards					
A/Hong Kong/4801/2014 (X-263B)	2016/109B	TGA	112	–	10 ± 1
A/Hong Kong/4801/2014 (X-263B)	Monovalent Bulk	Mfg.	341 ± 26	355 ± 35	37 ± 1

Sample ID	Type	Virus strain			Expected HA (µg/mL)
Samples analyzed					
H3 80	Monovalent Vaccine	A/Hong Kong/4801/2014 (X-263B)			80
H3 40	Monovalent Vaccine	A/Hong Kong/4801/2014 (X-263B)			40
H3 20	Monovalent Vaccine	A/Hong Kong/4801/2014 (X-263B)			20
H3 10	Monovalent Vaccine	A/Hong Kong/4801/2014 (X-263B)			10
H3 5	Monovalent Vaccine	A/Hong Kong/4801/2014 (X-263B)			5
H3 0	Negative Control				0
H3 FD	Degraded 80 µg/mL	A/Hong Kong/4801/2014 (X-263B)			17 ± 2 (by SRID)

SRID (single radial immunodiffusion assay); paBCA (purity adjusted bicinchoninic acid); IDMS (isotopic dilution mass spectrometry).

in-process samples as well as adjuvanted vaccines [17]. Although the results have not been published, the VaxArray platform performed well, in the large comparative studies sponsored by the U.S. Department of Health and Human Services (HHS), the National Institute for Biological Standards and Controls (NIBSC), and the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) [1].

The objectives of the study described herein were twofold: (i) to establish the relationship between HA measured by VaxArray and its immunogenicity, and (ii) to evaluate the performance of a new VaxArray assay for NA as a predictor of immunogenicity. The immunogenicity induced by HA was determined via the hemagglutination inhibition (HAI) assay and the immunogenicity induced by NA was determined by a neuraminidase inhibition (NAI) assay [18]. Neuraminidase inhibition titers have been shown to be well correlated with vaccine protection [10].

2. Materials and methods

2.1. Standards and samples

The standards and samples included in this study are listed in Table 1. Given the initiative to enable potency testing prior to the availability of reference reagents, a monovalent bulk (MB) intermediate was evaluated as a possible internal standard by comparing its response to that of a matched reference antigen distributed by the Therapeutics Goods Administration (TGA) in Australia. To characterize the MB, the matched TGA reference antigen was used in conjunction with the appropriate reference antisera to determine the potency of the MB by SRID. The MB was also characterized by a physiochemical method, as described below. Note that for simplicity the term "vaccine" is used to describe each of the concentrations of the monovalent A/H3N2 preparations that were made by sterile serial dilution of a known concentration of the MB. The force degraded sample was prepared from the 80 µg/mL vaccine. Specifically, samples were heated in a water bath for 20 h (T20) while a control was retained at 4 °C (T0). The water temperature was continuously monitored and was 55–56 °C during the entire degradation time period. After degradation, the vials were briefly cooled on ice and then stored at 4 °C until analysis later that day. Each vial was re-weighed before analysis to check for possible evaporation during degradation. All weights showed <0.07% difference after degradation.

2.2. Potency by SRID

The SRID assay was performed as described previously [19] with minor modifications. Specifically, each vaccine preparation was analyzed in 6 replicates randomly dispersed across 3 gels (two replicates of each vaccine preparation per gel). Two replicates of the standard curve were also analyzed on each gel, for a total of 6 replicates of each standard. Fig. 1 shows an example SRID gel after processing and staining.

2.3. Purity adjusted total protein (paBCA)

For the vaccine monobulk, 20 µL were denatured at 95° with 0.35% SDS and 55 mM DTT before being deglycosylated in the presence of PNGase F (V4831, Promega) overnight at 37 °C. The XCell *SureLock* gel box and NuPAGE™ pre-cast 4–12% bis-tris gradient gels (ThermoFisher NP0322) were used to evaluate the deglycosy-
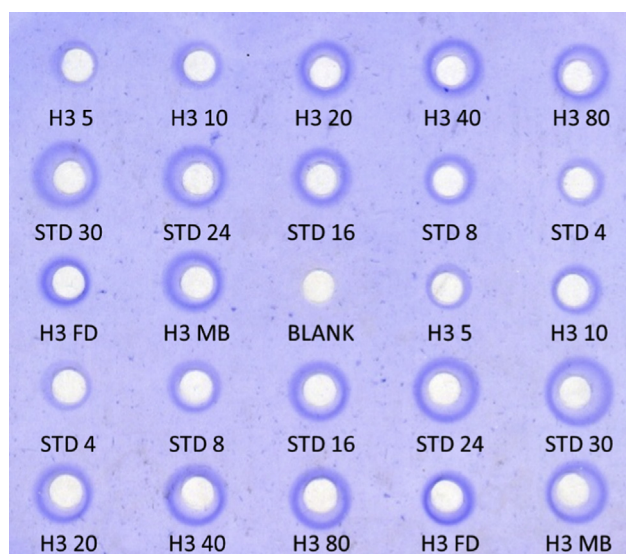

Fig. 1. A representative image of a SRID gel. A 5-point standard curve of reference antigen was analyzed in duplicate on each gel. Each sample, including the monovalent bulk (H3 MB), was lysed with 1% Zwittergent and diluted such that the expected HA content fell within the standard curve and was analyzed in duplicate. The values at the end of each label describe the expected HA concentration of the sample/standard. H3 FD is the forced degraded vaccine sample.

lated monobulk alongside non-treated monobulk to evaluate band shifts. The gel was stained with Coomassie® and imaged with an Olympus camera. Protein content of HA bands was calculated by applying the relative densitometric peak area of both HA1 and HA2 bands in the deglycosylated lanes to the total protein content determined by the micro bicinchoninic acid (BCA) assay (Thermo-Fisher 23235).

2.4. VaxArray assays

At the heart of the VaxArray Influenza Seasonal HA reagent kit (v1.2 VX-7150, InDevR Inc.) are microarrays containing a panel of subtype-specific anti-HA monoclonal antibodies. More information about the panel of antibodies on the microarray is available on the manufacturer's website and in the literature [16,17]. The microarrays within the VaxArray Influenza Seasonal NA reagent kit are composed of a panel of anti-NA mAbs licensed from the Icahn School of Medicine at Mount Sinai and more information may be found in published literature [20–22]. For each analysis time point and protein type (HA or NA), three VaxArray slides were removed from the refrigerator and equilibrated at room temperature for 30 min in their foil pouch. For quantification in this study, eight arrays were used for an 8-point calibration curve using TGA reference standards, eight arrays were used for an 8-point calibration curve using the monobulk solution as the reference standard, and the remaining 32 arrays were used for samples. The samples were processed by the method described in the VaxArray Operation Manual (R003). In short, standards and samples were diluted with phosphate buffered saline (PBS) and treated with 1% Zwittergent 3–14 for 30 min. Each standard was then serially diluted with Protein Blocking Buffer (PBB) (VX-6302, InDevR Inc.) with 1% Zwittergent (PBBZ) to make 8 calibration standards. PBBZ was also added to each sample to yield the final dilutions for analysis. After placing the slides in a humidity chamber, 50 µL of each standard was applied to wells on the left side of two slides, and 50 µL of each sample was added to the remaining wells in quadruplicate and incubated in a dark humidity chamber for one hour. The antigens were removed and 50 µL of a fluorescent detection label, consisting of a mix of the Fiducial Label and the Polyclonal A/B Label (VX-7601, InDevR Inc) in PBB, was applied and incubated for 30 min. An additional experiment was performed which incorporated a monoclonal fluorescent detection label (VX-7608, InDevR Inc) in place of the polyclonal fluorescent detection label. The fluorescent detection label mixture was removed with an 8-channel pipette and slides were sequentially washed with Wash Buffer 1 (VX-6303, InDevR Inc.), Wash Buffer 2 (VX-6304, InDevR Inc), 70% ethanol and 18 MΩ water using a wash bin. The water was removed using an air source and the back of each slide was washed with a tissue wetted with 70% ethanol, dried with a clean tissue wipe, and placed in a drying box for ∼10 min. Imaging was conducted on a VaxArray Imaging System (VX-6000, InDevR Inc.), which has LED excitation centered at 530 nm and fluorescence emission collection at 570 nm. Image collection times ranged from 200 ms to 1200 ms per array. Data was automatically processed using VaxArray Processing Workbooks described by Kuck et al. [16]. The linear ranges were automatically calculated, plotted and the HA (or NA) concentration measured in quadruplicate for each sample was automatically averaged. Samples were quantified against both the TGA and monobulk calibration curves.

2.5. Neuraminidase activity assay

The Neuraminidase Activity Assay was performed according to the product technical sheet for the Neuraminidase Activity Assay Kit from Sigma-Aldrich (MAK121). Briefly, a standard curve of standardized enzyme, provided in the kit, was serially diluted in water to 80, 48, 24, and 0 µM. Samples were diluted in water by weight. 20 µL of each sample was added to a clear flat-bottom, black walled optical grade 96-well plate (ThermoFisher 265301) in replicates of 6 and standards were added in triplicate. A reaction mix (with substrate) as well as a blank reaction mix (no substrate) was prepared. To half of the sample replicates and all of the standard replicates 80 µL of reaction mix was added. To the remaining half of the sample replicates 80 µL of the blank reaction mix was added.

The plate was sealed with optical grade film (ThermoFisher 4313663) and incubated in an Optima FLUOstar 96-well plate reader with a holding temperature of 37 °C with intermittent shaking. Every 120 s, every well of the plate was excited by LED at 530 nm and the associated fluorescent emission at 570 nm was measured. After 90 min, the plate was removed from the plate reader and discarded.

The fluorescent intensity of the replicate standard wells at t = 50 min was averaged and plotted against the corresponding standard concentrations. The standard curve was evaluated for linearity, the slope of the linear regression was calculated, and the activity of each sample was determined as described in the manufacturer's instructions. An average and standard deviation for the 3 replicates were calculated and the proper dilution factors were applied.

2.6. Determination of NA content by isotope dilution mass spectrometry (IDMS)

The concentration of NA in samples was measured by IDMS following published methods [23,24].

2.7. Immunization protocol

The immunization component of the study was performed at BioQual, Inc, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). This nonclinical laboratory study was based on generally accepted procedures for the immunization of biological compounds was conducted in full accordance with the United States Federal Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58. The immunizations were conducted in accordance with BioQual Standard Operating Procedures with approval by the Institutional Animal Care and Use Committee.

InDevR supplied BioQual with vials of vaccine formulations shipped on ice packs and provided documentation on proper storage and handling procedures prior to immunizations. Test articles for immunization were received at BioQual (Rockville, MD) from InDevR in a blinded fashion and immunized in mice in a blinded manner. Serum samples collected from immunized mice were labelled according to the nomenclature identical to the code provided on the test article label.

Inbred female mice of the BALB/cAnNHsd background were obtained from Envigo (Frederick, MD). The animals were approximately 6 to 8 weeks of age at arrival at BioQual. This study was designed to use the fewest number of animals possible that would allow sufficient group sizes for meaningful statistical analysis of data, consistent with the objective of the study, the scientific needs of the Sponsor, contemporary scientific standards, and in consideration of applicable regulatory requirements.

The vaccine formulations were administered intramuscularly (IM). The syringes (0.25 ml syringe, 1 syringe/mouse) were filled up with 50 µL of the vaccine formulation, just prior to the immunization, and stored on ice prior to injection. Injection was administered as soon as possible and within 1hr of preparation of the syringes. Each mouse received the vaccine as an IM injection in 50 µL volume into one or both hind thighs. However, during the

prime round of injections 20% of the mice received less than the full antigen dose due to an error in the fill volume. Since the study involved both prime and boost immunizations and no dose delivery errors were made in the boost round, none of these animals were excluded from the study. All mice in the group (5 for each concentration) received immunizations on study Day 1 and study Day 21 and were terminally bled on study Day 42.

Approximately 0.5 ml of terminal blood samples were collected into BD Vacutainer SST, incubated at room temperature for at least 30 min, centrifuged at ~3000 rpm for 20 min and aliquoted. Serum was collected, aliquoted into two 0.5 ml sterile cryovials per sample, and stored at −80 °C. Vials were labeled with animal number, collection date, and dose group designation supplied by InDevR.

2.8. Hemagglutination inhibition (HAI) assay

Aliquots of mouse sera were stored at −20 °C (nominal temperature) and used in HAI assays following a standard protocol [25]. Chicken allantoic fluid containing A/Hong Kong/4801/2014 was titrated for agglutination of turkey red blood cells, and a dilution containing 4 hemagglutinating units (HAU)/25 μL, used in the assay. Serum samples underwent a standard receptor destroying enzyme treatment at 37 °C overnight followed by inactivation at 56 °C for 30 min. The sera were then adsorbed with packed turkey red blood cells (RBCs) to remove non-specific agglutinins. Individual RDE-treated serum samples at an initial 1:10 dilution were dispensed into the first set of wells and then serially (2-fold) diluted across the plate. Each plate included non-agglutinated negative controls containing neither serum nor virus. The plate was incubated at room temperature for 30 min to allow antibody-virus binding. After incubation, 0.5% turkey RBCs were added to each well and the plate was incubated at room temperature for 45 min. All plates were first imaged in the Cypher One (CY-6000, InDevR Inc) system (~40 s) and then immediately transferred to

the experienced human reader to determine the HAI titer based on tear drop formation when the plates were tilted at a 45 degree angle. The reciprocal of the last serum dilution that inhibited agglutination was recorded as the HAI antibody titer.

2.9. NA inhibition (NAI) assay

NAI antibody titers were measured by enzyme-linked lectin assay (ELLA) following a published method [26]. $H6N2_{HK/14}$ reassortant virus was used as the source of antigen in ELLA to overcome non-specific inhibition by HA-specific antibodies. The reassortant influenza virus contains the HA (H6) gene from A/turkey/Massachusetts/3740/1965, the NA gene of A/Hong Kong/4801/14 (H3N2), and all other gene segments from A/PR/8/34 (H1N1), and was generated by reverse genetics as described previously [27,28]. The sera were incubated at 56 °C for 45 min and then serially (2-fold) diluted in a 96 well plate prior to transferring to fetuin-coated 96-well plates. A dilution of $H6N2_{HK/14}$ virus that resulted in 90% of maximum signal (virus alone) was then added and the plate incubated for 18–20 hr at 37 °C. After washing the plate, HRP-conjugated peanut agglutinin (Sigma-Aldrich, St. Louis, MO) was added and the plate incubated at room temperature for 2 hr. Substrate (OPD) was added after washing the plate. The reaction was stopped after 10 min incubation. OD_{490} values were measured and the percent inhibition calculated after subtracting background values. The inverse of the dilution that resulted in ≥50% inhibition was reported as the NAI antibody titer.

3. Results and discussion

3.1. Microarray layout and processing

Schematics for the VaxArray HA and NA microarrays are shown in Fig. 2 along with representative fluorescence images for the TGA reference antigen and the monobulk intermediate. Unless otherwise noted, in all cases a fluor-conjugated polyclonal ("universal") antibody from InDevR (VX-7601) served as the fluorescent detection label in the VaxArray sandwich assay. Note that for the array of anti-HA mAbs ("HA array") only the H3(i) capture mAb yielded a response, as expected for this particular vaccine. For the array of anti-NA mAbs ("NA array"), both N2 capture mAbs were responsive. The prototype version of the NA array used in this study also contained a single capture mAb against NP but all signals for the reference antigen were saturated at the concentrations relevant for analysis of NA so no results are reported. However, it is worth noting that the NP signal for the subunit vaccine was quite low, as expected. Fluorescence signals for each printed antibody (9 replicates per mAb) were extracted and processed in the manufacturer's software, as previously described [16].

3.2. Reference antigens and calibrants for VaxArray

As with SRID, the VaxArray potency test is not an absolute method and therefore requires a known standard for quantification of an unknown. The reference reagents for SRID, both antigens and antisera, are generated, qualified, and distributed by the network of Essential Regulatory Laboratories (ERLs). The HA concentration of a Primary Liquid Standard (PLS), which is generally a whole, inactivated virus, is determined by purity adjusted total protein measurement at multiple Essential Regulatory Laboratories (ERLs) [29]. The PLS is subsequently used as the standard in SRID to determine the HA content of the reference reagent. The process of generating and qualifying the reference reagents can take months, with reference reagents typically made available after manufactur-
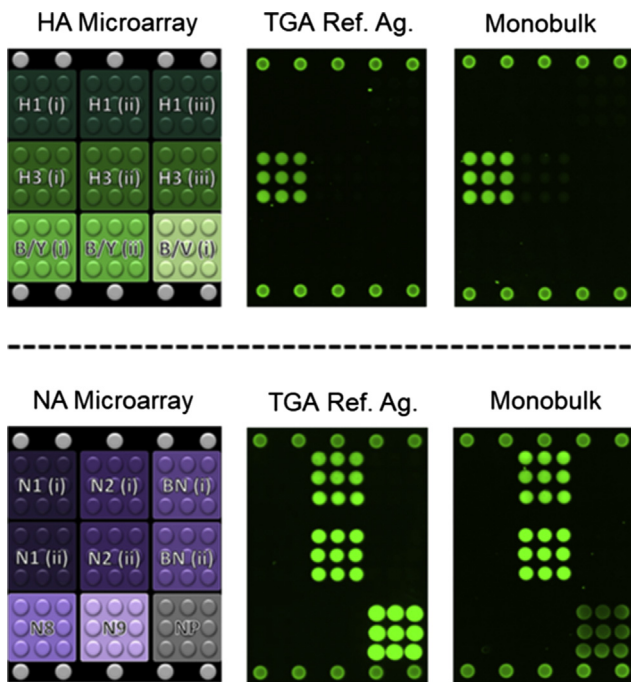

Fig. 2. The upper panel illustrates the HA microarray layout (far left image) along with representative fluorescence images for the H3 reference antigen and monobulk intermediate. The 5 fluorescent spots at the top and bottom of each array are fiducial markers. The lower panel illustrates the NA microarray layout (far left) along with representative fluorescence images for the N2 reference antigen and monobulk intermediate.

ers have initiated vaccine production at risk. Vaccine producers generally take on this risk in order to meet the demanding timeline for delivering flu vaccine prior to the start of a new season.

One step toward streamlining vaccine production is an alternative potency assay that could be reliably used in combination with an internal standard generated by the vaccine producer during early test batches. Such a system could be useful for early quantification of in-process samples, such as yield assessment in crude harvest, or antigen recovery after purification. In this context, reliability is defined by the ability to yield a SRID-equivalent or correlated result. If the internal standard were sufficiently robust, it is possible that the stability of monovalent formulations could be tracked during storage prior to the availability of the ERL-released reference reagents.

Therefore, in this study we evaluated an ERL qualified reference antigen from TGA as well as an internal standard from the vaccine manufacturer as potential calibrants for VaxArray. The internal standard itself was calibrated in two ways and the results are summarized in Table 1. In consideration of early development in-process testing, the internal standard was calibrated using the same approach that is employed for the Primary Liquid Standard – purity adjusted total protein, an absolute method that can be applied before reference reagents are available. The paBCA determined HA concentration for the MB with one standard deviation was $355 \pm 35 \, \mu g/mL$. The MB was also calibrated in SRID against the TGA reference antigen and antisera, which yielded a value $341 \pm 26 \, \mu g/mL$ (error is one standard deviation) Within error, these two values are equivalent.

For calibration of the VaxArray NA assay, the NA total protein content of the TGA reference antigen and the MB formulation was determined by IDMS. In both cases, the mass concentration of NA was approximately 10x less than the mass concentration for HA (e.g., $37 \pm 1 \, \mu g/mL$ of NA in the MB relative to $341 \, \mu g/mL$ of HA). These results are within expectations based on previous work [9]. These total NA protein values were used to calibrate the VaxArray NA assay for quantification of the NA content within each of the vaccine preparations.

3.3. Comparison of calibrant performance in VaxArray HA

Serial dilutions of both calibrants in the VaxArray HA potency assay are shown in the upper panel (A) of Fig. 3. The standards were evaluated over the same concentration range, based on their original SRID-determined concentrations. Note that the concentration range is as measured on the array and that, due to the high sensitivity of the VaxArray assay, large dilution factors were needed for these concentrated antigens. It is clear that both calibrants exhibit the same sensitivity (slope). The lower panel (B) of Fig. 3 shows a direct comparison of the linear regression slopes for each calibrant, including the slope obtained for the paBCA-calibrated MB standard. Within error, both standards exhibit an equivalent response, even when the MB is calibrated by paBCA. Thus, either calibrant could be used in VaxArray. For the MB as calibrant, the standard value could be determined by paBCA or by SRID.

3.4. Comparison of SRID and VaxArray measured potency

The potency of each of the five vaccine concentrations along with the force degraded sample was determined by SRID, using the TGA reference antigen and appropriate antisera, and by VaxArray using both the TGA reference antigen and the MB as calibrants. Potency measurements were made on Day 1 and on Day 21 for VaxArray, with sample preparation and testing completed within a few hours on each day. The SRID assay required two days but was initiated on Day 1 and Day 21 as well. The upper left panel
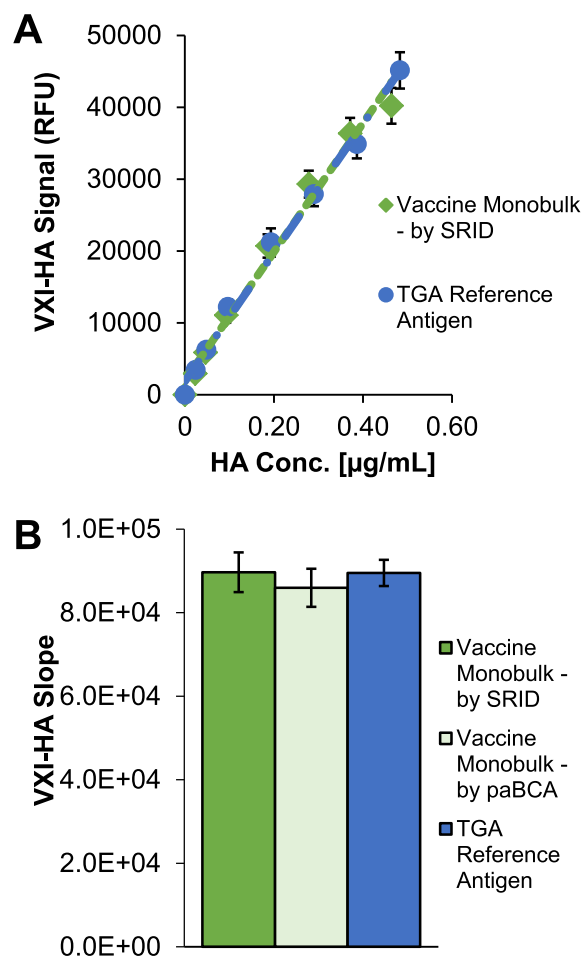

Fig. 3. (A) Calibration curves on the H3(i) capture antibody with pAb label from Day 21 for the MB and TGA reference antigen. The x-axis concentration was based on the assigned SRID values for each calibrant with the assumption of accurate serial dilution. The VaxArray signal on the y-axis is designated by VXI-HA. Error bars represent the standard deviation across the 9 antibody spots in each array. A linear regression was applied through all 8 calibration points for each curve to calculate slope and error in the slope. (B) Comparison of the VaxArray slopes for each calibrant and method of calibration. Error bars represent the error in the slope for each curve.

(A) of Fig. 4 shows the correlation between the average VaxArray determined HA potency and the average SRID value when VaxArray was calibrated using the MB as a standard and the pAb label is used. With the exception of the force degraded sample, the correlation between VaxArray and SRID was quite good ($R^2 = 1.00$), as expected since the MB was calibrated by SRID. The red symbol in the graph represents the force degraded sample and was not included in the correlation analysis. For VaxArray's capture mAb H3(i), the sandwich assay with a polyclonal fluorescent detection label is not as highly stability indicating as SRID. However, as shown in the upper right panel (B), when an H3-specific monoclonal fluorescent detection label is used the VaxArray values were more closely aligned with the SRID value. This is consistent with previous studies that have suggested that monoclonal fluorescent detection labels are often more sensitive to changes in protein confirmation than polyclonal fluorescent detection labels.

All measured potency values are plotted for side-by-side comparison in the lower panel (C) of Fig. 4 for all calibration approaches. Note that the $5 \, \mu g/mL$ vaccine was below the limit of detection for SRID but easily quantified by VaxArray. The individual points represent Day 1 and Day 21 potency measurements. For each potency assay and calibrant combination, the average
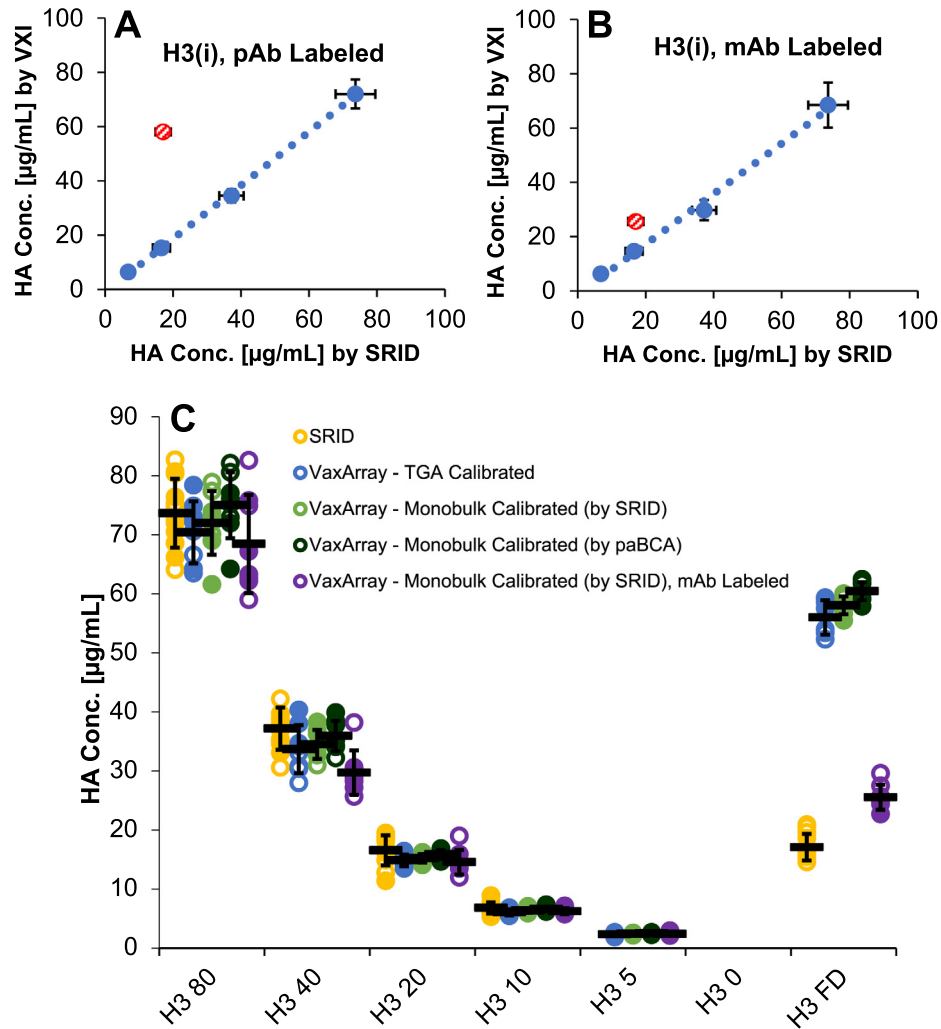
Fig. 4. Comparison of vaccine potency measured by SRID and VaxArray HA. (A) shows the correlation between average values when the mAb label was used for Vaxarray. (B) shows the correlation between average values when the pAb label was used for Vaxarray. In both cases, the vaccine monobulk was used as the calibrant for VaxArray. The forced degraded sample is shown as a red, striped point on both plots. Error bars represent the standard error across all replicates analyzed. Linear regression to all of the data yielded the following parameters for (A) y = 1.00x − 1.42 with an R^2 of 1.00 and for (B) y = 0.93x − 1.44 with an R^2 of 0.99. Panel (C) displays the individual potency measurement for all of the calibrants and calibration methods. Open circles are replicates (6) analyzed on day 1; closed circles are replicates (6) analyzed on day 21. Solid bars represent the average of all day 1 and day 21 replicates, along with the associated standard deviation as error bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and standard deviation representing all replicates is shown as solid dash with error bars. Note that for vaccine samples with expected concentrations of 80–10 µg/mL, the potency values were equivalent within the measurement error for both assays. Two conclusions can be drawn from this data: (i) VaxArray can be accurately calibrated with an ERL-reference antigen as well as an internal standard, and (ii) with the exception of the force degraded sample, VaxArray is not only highly correlated with SRID, the values are equivalent – even when the internal standard was characterized by paBCA. These results support the idea that an internal standard could be used in combination with VaxArray to track potency during early vaccine production efforts.

3.5. Comparison of NA enzymatic activity and VaxArray measured potency

Although there is no gold standard for quantification of NA in flu vaccines, and its level is not standardized, it is recommended that vaccine producers demonstrate its presence at detectable levels using an appropriate assay [14,15]. The performance of the VaxArray NA assay was therefore compared to the activity as

measured by a Neuraminidase Activity Assay utilizing a MUNANA-like (2-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid) substrate for all of the vaccine formulations included in this study. The top panel (A) of Fig. 5 is a correlation plot of the average results for each assay. Importantly, the NA concentration for the force degraded sample was not detectable by either method, indicating that both methods are sensitive to changes in NA protein structure and can be used to assess NA stability.

The lower panel (B) of Fig. 5 is a plot of the individual VaxArray NA data points as a function of expected concentration (defined by HA concentration). When the NA content of the TGA reference antigen was used to calibrate the system, the values were slightly lower than when the MB NA content (both measured by IDMS) was used to calibrate the system. However, the values are equivalent within measurement error and for both calibrants the concentration for NA was ~10x less than the mass concentration of HA.

3.6. Immunogenicity

The individual titer values plotted in Fig. 6 are the results from sera collected at Day 42, after both prime and boost immuniza-
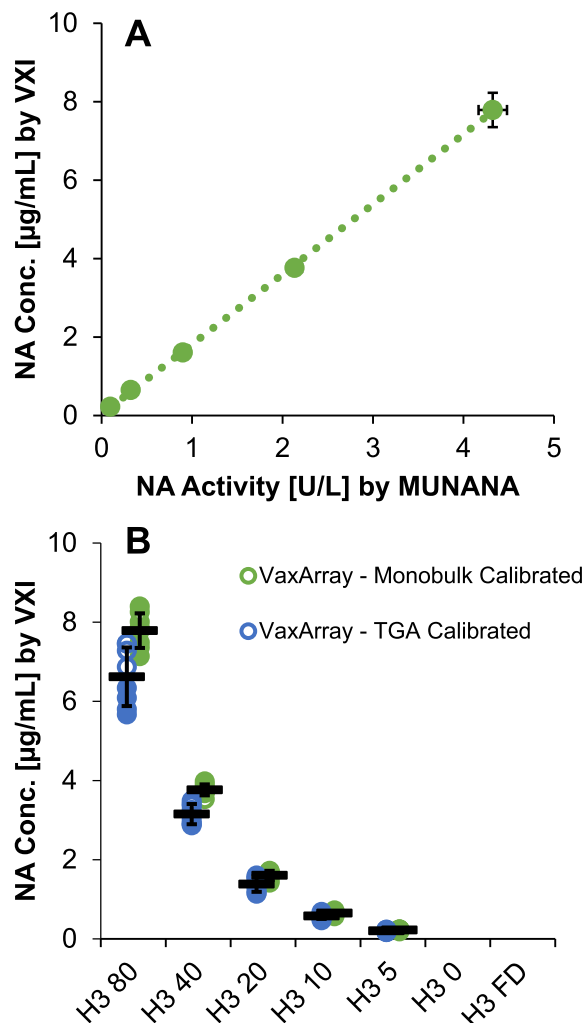
Fig. 5. (A) is a correlation plot of the overall average NA N2 potency measured by VaxArray from Day 1 and Day 21 combined and NA activity measured by MUNANA. Error bars represent the standard error across the replicates analyzed. VaxArray NA was calibrated using the IDMS value for the MB. A linear regression to data yields y = 1.79x + 0.03 with an R^2 of 1.00 (green dotted line). (B) shows the individual VaxArray measurements for all 8 replicates. Open circles represent replicates analyzed on day 1; closed circles represent replicates analyzed on Day 21. Solid bars represent the average of all Day 1 and 21 replicates with standard error. All replicates analyzed for H3 0 and H3 FD were below the limit of quantification of the VXI assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tions. The left panel (A) of Fig. 6 is the data from the HAI assay and the right panel (B) is the data from the NAI assay (ELLA). For the ELLA assay, each sample was analyzed in duplicate. Note that the y-axis scale for both graphs is linear and that the solid bars represent the geometric mean titer. Titer values for the force degraded samples are highlighted within a gray box. A negative control was included in the study (designated as H3 0), with 5 mice receiving 50 μL of sterile PBS. The mice that received the negative control exhibited a non-zero titer as measured by both assays. None of the potency assays (SRID, VaxArray HA and NA, MUNANA) exhibited false positives for the negative control, including a post-study investigation of residual materials returned to InDevR from BioQual. The study did not include a pre-immunization sample from the mice so it was not possible to determine the source of contamination or pre-exposure. For the HAI assay, the force degraded sample exhibited an immune response similar to that of the 20 μg/mL vaccine formulation, which is qualitatively consistent with the

SRID and VaxArray (with mAb fluorescent detection label) measured potency (see Fig. 4). It is especially encouraging to note that the forced degraded sample did not illicit a measureable anti-NAI immune response, consistent with the VaxArray NA potency assay which indicated non-detectable levels of intact NA.

3.7. VaxArray potency as predictor for HA and NA induced immunogenicity

According the Code of Federal Regulations (21 CFR 610.10 and 21 CFR 600.3(s)), the results from a potency test should accurately reflects a product's ability to effect a given clinical result [30,31]. The SRID potency test has served this purpose for the HA content of flu vaccines, with an accepted relationship between SRID measured potency and clinical immunogenicity as measured by the HAI assay [1]. To evaluate the potential of the VaxArray HA and NA potency assays to serve as a proxy for immunogenicity, the GMT titers for HAI (left panel, **A**) and NAI (right panel, **B**) are plotted against measured potency in Fig. 7 for both SRID and VaxArray. Qualitatively, the trends for both HAI and NAI data sets are strikingly similar, with a sigmoidal function representing higher induced immunogenicity at higher antigen dose up to a limiting dose. The data shown in Fig. 7A includes 4 values for the dose response measured by SRID (since the lowest concentration vaccine was below the LOD) as well as the force degraded sample, for a total of 5 points. The VaxArray data set shown in Fig. 7A represents the H3(i) capture mAb with the mAb fluorescent detection label using the MB calibrant. Including the force degraded sample, the VaxArray data set has 6 points. All of the points along both curves were fit with the following equation using non-linear regression [32]:

$$y = \frac{c}{1 + e^{b-ax}} \tag{1}$$

where **a** is the rise rate, **b** is the inflection point, and **c** is the asymptote. It is clear from the non-linear regression that the function fits well. To quantitatively evaluate the trends, the 95% confidence intervals (CI) for the HAI-SRID data set were determined by a Monte Carlo method [33] and compared to the fit parameters for the VaxArray data. The rise rate (**a**) from non-linear regression to the SRID data set is 0.084, with a lower CI of 0.059 and an upper CI of 0.110. The rise rate for the VaxArray data set was 0.078, well within the 95% confidence interval.

The NAI data as a function of VaxArray measured NA concentration (Fig. 7B) is also fit well to the non-linear function (Eq. (1)) and the relationship is predictive. Thus, it is reasonable to conclude that the VaxArray NA assay measures potency and can serve as a proxy for immunogenicity.

3.8. Future considerations

While the VaxArray assays have been developed to be fairly resistant to evolutionary change by probing more than one relatively conserved epitope for each subtype, it is possible that a new strain could arise that the mAb panel for a given subtype fails to detect. In the event that a new strain is not detected on the current version of the array, InDevR has developed a performance management plan with specific protocols to rapidly screen a substantial archive of anti-HA mAbs in a highly multiplexed format. If an appropriate mAb is identified, it can be incorporated into the array, verified and validated in an expedient, but quality managed, process. A concrete example of this process occurred after the strain selection committee within WHO announced this year new strains for A/H3 and B/Victoria-like viruses to be included in the 2018/2019 flu vaccines for North America. These strain changes necessitated new mAbs for improved sensitivity. Accordingly,
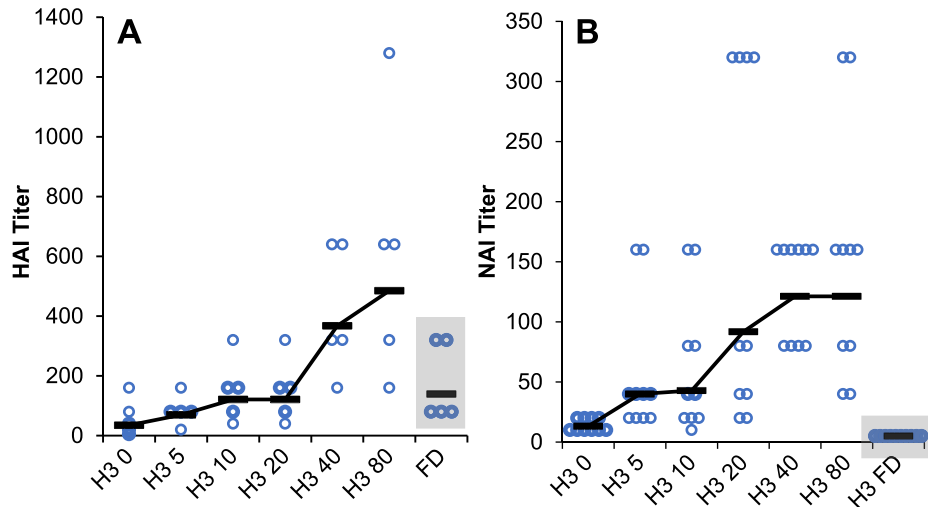
Fig. 6. (A) Hemagglutination inhibition titers as a function of vaccine sample. (B) Neuraminidase inhibition titers as a function of vaccine sample. The geometric mean titer of each vaccine is shown as solid bars connected by a solid line. The forced degraded sample is shown in a gray-shaded box for both assays.
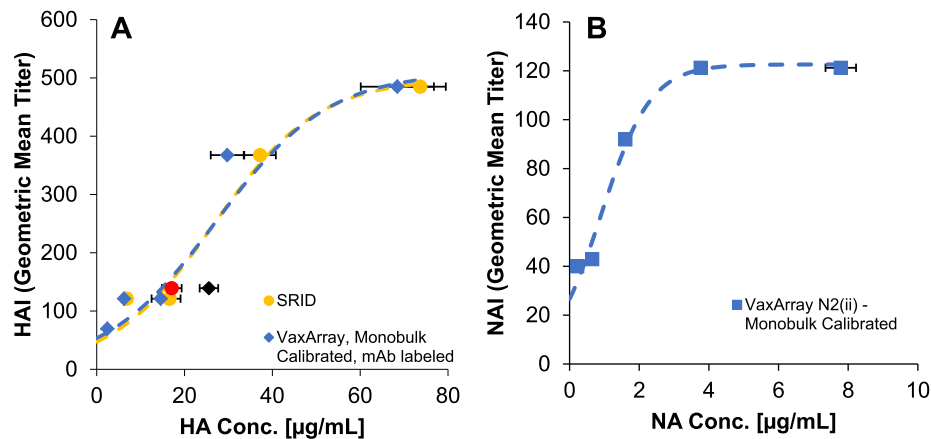

Fig. 7. (A) The geometric mean HAI titer is plotted against the associated average (of all replicates of Day 1 and Day 21) HA concentration as determined by SRID (gold circles) and VaxArray (blue diamonds). The force degraded sample for SRID is highlighted by a red circle and by a black diamond for VaxArray. The blue and gold dotted lines represent a non-linear regression to a 3-parameter logistic function for VaxArray and SRID, respectively. (B) The geometric mean NAI titer is plotted against the average (of all replicates of Day 1 and Day 21) NA concentration as determined by VaxArray. The dotted lines represent a non-linear regression to a 3-parameter logistic function. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

InDevR implemented the performance management plan, screened and qualified existing mAbs, and released a new version of the microarray within 8 weeks [34]. Of course, if no mAb exists for a particular subtype change, the normal process for developing a responsive mAb would be followed.

4. Conclusions

The results of this study demonstrate that when calibrated appropriately, the VaxArray Influenza Seasonal HA potency assay yields SRID-equivalent results. The VaxArray NA assay is strongly correlated with enzymatic activity, is highly sensitive to immunogenic forms of the glycoprotein, and is correlated with serum levels of anti-NAI antibodies as measured by neuraminidase inhibition. Based on the performance of both tests, it should be possible to use an internal standard defined by paBCA or IDMS in conjunction with VaxArray to track HA and NA potency early in vaccine development. Although more testing is needed, the consistent and "predictive" immunogenicity-potency relationships observed for VaxArray HA and NA support a conclusion that these assays are indeed a measure of each antigen's potency and can serve as a

proxy for immunogenicity. While this study was conducted with a monovalent vaccine, the VaxArray assays are multiplexed and enable simultaneous analysis of all antigen subtypes within multivalent vaccines.

Senior authors' information

Dr. Kuck is a former Science Advisor for the USFDA and co-founder of InDevR. She now serves as scientist and Executive Vice President at InDevR. Dr. Eichelberger was a Principal Investigator in the Division of Viral Products, Office of Vaccines Research and Review from 2006to 2017 and is now the Director of the Division of Biological Standards and Quality Control, Office of Compliance and Biologics Quality, CBER, US-FDA. Dr. Rowlen is a former Professor of Chemistry (University of Colorado in Boulder from 1991 to 2008) and co-founder. She now serves as the Chief Executive Officer and Chief Science Officer at InDevR.

Funding source

Acknowledgements

References

[1] Wood JM, Weir JP. Standardisation of inactivated influenza vaccines-Learning from history. Influenza Other Respi Viruses 2018. https://doi.org/10.1111/irv.12543.

[2] Minor PD. Assaying the potency of influenza vaccines. Vaccines 2015;3:90–104. https://doi.org/10.3390/vaccines3010090.

[3] Thompson CM, Petiot E, Lennaertz A, Henry O, Kamen AA. Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. Virol J 2013;10:141. https://doi.org/10.1186/1743-422X-10-141.

[4] Ross TM, Mahmood K, Crevar CJ, Schneider-Ohrum K, Heaton PM, Bright RA. A trivalent virus-like particle vaccine elicits protective immune responses against seasonal influenza strains in mice and ferrets. PLoS One 2009;4:2–11. https://doi.org/10.1371/journal.pone.0006032.

[5] Cox MMJ, Patriarca PA, Treanor J. FluBlok, a recombinant hemagglutinin influenza vaccine. Influenza Other Respi Viruses 2008;2:201–9. https://doi.org/10.1111/j.1750-2659.2008.00053.x.

[6] Venereo-Sanchez A, Gilbert R, Simoneau M, Caron A, Chahal P, Chen W, et al. Hemagglutinin and neuraminidase containing virus-like particles produced in HEK-293 suspension culture: An effective influenza vaccine candidate. Vaccine 2016;34:3371–80. https://doi.org/10.1016/j.vaccine.2016.04.089.

[7] Shoji Y, Chichester JA, Jones M, Manceva SD, Damon E, Mett V, et al. Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. Hum Vaccin 2011;7:41–50. https://doi.org/10.4161/hv.7.0.14561.

[8] Rajendran M, Nachbagauer R, Ermler ME, Bunduc P, Amanat F, Izikson R, et al. Analysis of anti-influenza virus neuraminidase antibodies in children, adults, and the elderly by ELISA and enzyme inhibition: evidence for original antigenic sin. MBio 2017;8:e02281–e2316. https://doi.org/10.1128/mBio. 02281-16.

[9] Wohlbold TJ, Krammer F. In the shadow of hemagglutinin: a growing interest in influenza viral neuraminidase and its role as a vaccine antigen. Viruses 2014;6:2465–94. https://doi.org/10.3390/v6062465.

[10] Memoli MJ, Shaw PA, Han A, Czajkowski L, Reed S, Athota R, et al. Evaluation of antihemagglutinin and antineuraminidase antibodies as correlates of protection in an influenza A/H1N1 virus healthy human challenge model. MBio 2016;7:e00417–e516. https://doi.org/10.1128/mBio. 00417-16.

[11] Bodewes R, Geelhoed-Mieras MM, Wrammert J, Ahmed R, Wilson PC, Fouchier RAM, et al. In vitro assessment of the immunological significance of a human monoclonal antibody directed to the influenza a virus nucleoprotein. Clin Vaccine Immunol 2013;20:1333–7. https://doi.org/10.1128/CVI.00339-13.

[12] Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J Immunol 2008;181:4168–76.

[13] Krammer F, Fouchier RAM, Eichelberger MC, Webby RJ, Shaw-saliba K. NAction! how can neuraminidase-based immunity contribute to better influenza virus vaccines ? Am Soc Microbiol 2018;9:1–12.

[14] European Medicines Agency. Guideline on influenza vaccines – quality module 2014.

[15] World Health Organization. Annex 3 Recommendations for the production and control of influenza vaccine (inactivated); 2005.

[16] Kuck LR, Sorensen M, Matthews E, Srivastava I, Cox MMJ, Rowlen KL. Titer on chip: New analytical tool for influenza vaccine potency determination. PLoS One 2014;9:e109616. https://doi.org/10.1371/journal.pone.0109616.

[17] Kuck LR, Saye S, Loob S, Roth-Eichhorn S, Byrne-Nash R, Rowlen KL. VaxArray assessment of influenza split vaccine potency and stability. Vaccine 2017;35:1918–25. https://doi.org/10.1016/j.vaccine.2017.02.028.

[18] Eichelberger MC, Couzens L, Gao Y, Levine M, Katz J, Wagner R, et al. Comparability of neuraminidase inhibition antibody titers measured by enzyme-linked lectin assay (ELLA) for the analysis of influenza vaccine immunogenicity. Vaccine 2016;34:458–65. https://doi.org/10.1016/j.vaccine.2015.12.022.

[19] Eichelberger SL, Sultana I, Gao J, Getie-Kebtie M, Alterman M, Eichelberger MC. Potency under pressure: the impact of hydrostatic pressure on antigenic properties of influenza virus hemagglutinin. Influenza Other Respi Viruses 2013;7:961–8. https://doi.org/10.1111/irv.12102.

[20] InDevR I. InDevR licenses Mount Sinai influenza antibodies for VaxArray neuraminidase potency assay | EurekAlert! Science News 2017. https://www.eurekalert.org/pub_releases/2017-09/ii-ilm091817.php [accessed April 10, 2018].

[21] Wohlbold TJ, Chromikova V, Tan GS, Meade P, Amanat F, Comella P, et al. Antibodies protect against Lethal H10N8 influenza virus infection in mice. Jvi 2016;90:851–61. https://doi.org/10.1128/JVI.02275-15.Editor.

[22] Wohlbold TJ, Podolsky KA, Chromikova V, Kirkpatrick E, Falconieri V, Meade P, et al. Broadly protective murine monoclonal antibodies against influenza B virus target highly conserved neuraminidase epitopes. Nat Microbiol 2017;2:1415–24. https://doi.org/10.1038/s41564-017-0011-8.

[23] Williams TL, Luna L, Guo Z, Cox NJ, Pirkle JL, Donis RO, et al. Quantification of influenza virus hemagglutinins in complex mixtures using isotope dilution tandem mass spectrometry. Vaccine 2008;26:2510–20. https://doi.org/10.1016/j.vaccine.2008.03.014.

[24] Williams TL, Pirkle JL, Barr JR. Simultaneous quantification of hemagglutinin and neuraminidase of influenza virus using isotope dilution mass spectrometry. Vaccine 2012;30:2475–82. https://doi.org/10.1016/j.vaccine.2011.12.056.

[25] Harris K, Ream R, Gao J, Eichelberger MC. Intramuscular immunization of mice with live influenza virus is more immunogenic and offers greater protection than immunization with inactivated virus. Virol J 2011;8:251. https://doi.org/10.1186/1743-422X-8-251.

[26] Couzens L, Gao J, Westgeest K, Sandbulte M, Lugovtsev V, Fouchier R, et al. An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera. J Virol Methods 2014;210:7–14. https://doi.org/10.1016/j.jviromet.2014.09.003.

[27] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci 2000;97:6108–13. https://doi.org/10.1073/pnas.100133697.

[28] Sandbulte MR, Gao J, Straight TM, Eichelberger MC. A miniaturized assay for influenza neuraminidase-inhibiting antibodies utilizing reverse genetics-derived antigens. Influenza Other Respi Viruses 2009;3:233–40.

[29] WHO Expert Committee on Biological Standardization. Annex 5: Generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories 2013. http://www.who.int/biologicals/areas/vaccines/TRS_979_Annex_5.pdf.

[30] Food and Drug Adminstration, Department of Health and Human Services, Subchapter F- Biologics. 21 CFR 600.3; 2017.

[31] Food and Drug Adminstration, Department of Health and Human Services, Subchapter F- Biologics. 21 CFR 610.10; 2017.

[32] Brown AM. A step-by-step guide to non-linear regression analysis of experimental data using a Microsoft Excel spreadsheet. Comput Methods Programs Biomed 2001;65:191–200. https://doi.org/10.1016/S0169-2607(00)00124-3.

[33] Hu W, Xie J, Chau HW, Si BC. Evaluation of parameter uncertainties in nonlinear regression using Microsoft Excel Spreadsheet. Environ Syst Res 2015;4:4. https://doi.org/10.1186/s40068-015-0031-4.

[34] Rodriguez S. VaxArray Rapid Response to Seasonal Strain Changes for Influenza Vaccines 2018. https://indevr.com/2018/04/vaxarray-rapid-response-seasonal-strain-changes-influenza-vaccines/ [accessed October 4, 2018].