VaxArray potency assay for rapid assessment of “pandemic” influenza vaccines

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The VaxArray Influenza Pandemic HA (VXI-pHA) potency assay is a multiplexed sandwich immunoassay that consists of nine broadly reactive yet subtype-specific monoclonal capture antibodies printed in microarray format and a suite of fluor-labeled secondary antibodies that were selected to probe conserved HA epitopes. VXI-pHA was designed to optimize the probability that the ready-to-use assay would work for the most concerning, emergent influenza A strains, eliminating the need for the time-consuming process of reference reagents production. The performance of this new potency test was evaluated using a panel of 48 potentially pandemic strains of influenza viruses and vaccines spanning 16 years of antigenic drift, including the most recent pre-pandemic vaccine being developed against the “5th wave” A/H7N9 virus. The VXI-pHA assay demonstrated coverage of 93%, 92%, and 100% for H5, H7, and H9 antigens, respectively. The assay demonstrated high sensitivity with linear dynamic ranges of more than 150-fold and quantification limits ranging from 1 to 5 ng/mL. For three production lots of H7N9 monobulk drug substance, the assay exhibited excellent accuracy (100 ± 6%) and analytical precision (CV 6 ± 2%). The high assay sensitivity enabled robust detection and quantification of hemagglutinin in crude in-process samples and low-dose, adjuvanted vaccines with an accuracy of 100 ± 10%.

RESULTS

The VXI-pHA assay was previously developed to provide broad coverage and highly sensitive detection of potentially pandemic influenza A H5, H7, and H9 subtype virus HA. The resulting assay, VXI-pHA, is a multiplexed sandwich immunoassay that consists of subtype-specific monoclonal antibodies on a microarray platform that capture HA protein before a fluor-labeled secondary antibody is added for detection (Fig. 1a). The subtype-specific monoclonal antibodies are printed in array format (Fig. 1b) in replicates of 9 on a 16-well glass slide. In work done prior to the studies outlined in this manuscript, a large panel of monoclonal antibodies licensed from the CBER was screened for coverage and sensitivity (data not shown). A minimum of two different mAbs was selected for each subtype to optimize coverage within a given subtype (Fig. 1b). The H5(i–iii) antibodies, with broad detection of pre-2014 H5 antigens...
do not detect the recently emerged H5N8 A/Gryfalcon-like strains (Fig. 1d) from Clade 2.3.4.4. For this reason, we included the H5(iv) and H5(v) antibodies, which were raised against A/Gryfalcon-like HA antigens. VXI-pHA has high coverage and specificity for H5, H7, and H9 antigens produced across multiple production platforms. A panel of 48 antigens spanning the H5, H7, and H9 subtypes was tested in the VXI-pHA assay at relatively high concentrations (5 µg/mL for recombinant HA (rHA) and 0.5 µg/mL for all other antigens) to evaluate coverage within a subtype, specificity for a specific subtype, and relative sensitivity. These antigen concentrations were selected because previously reported studies found that rHA antigens are more compatible with the VaxArray platform at slightly higher concentrations than egg-derived antigens. Representative fluorescence images for the following vaccines on the VXI microarray: a) A/Vietnam/1203/2004 from H5 Clade 1 (egg derived, CBER Reference Antigen, Lot #50), d) A/Gryfalcon/Washington/41088-6/2014 from H5 Clade 2.3.4.4 (egg derived, IRR, Cat # FR-1447), e) A/Guangdong/17SF003/2016 from the H7N9 subtype (rHA), and f) A/chicken/Hong Kong/G9/1997 from H9 Clade G1 (NIBSC, Lot # 08/228).

**Table:**

| mAb ID | Epitope Type | HAI activity | Detection Target |
|--------|--------------|--------------|------------------|
| H5 (i) | Conformational | YES | Broad H5 |
| H5 (ii) | Conformational | YES | Broad H5 |
| H5 (iii) | Conformational | YES | Broad H5 |
| H5 (iv) | Conformational | YES | A/Gryfalcon-like, Clade 2.3.4.4 |
| H5 (v) | Conformational | YES | A/Gryfalcon-like, Clade 2.3.4.4 |
| H7 (i) | Conformational | YES | Broad H7 |
| H7 (ii) | Conformational | YES | Broad H7 |
| H9 (i) | Conformational | YES | Broad H9 |
| H9 (ii) | Conformational | YES | Broad H9 |

**Fig. 1** VaxArray influenza pandemic HA potency assay (VXI-pHA). a) Illustration of the immunoassay principle. b) Schematic of the VXI-pHA array layout of subtype-specific antibodies to A/H5, A/H7, and A/H9 subtypes. The array contains nine replicate spots (~200 µm in diameter) of each monoclonal antibody. Table provides epitope information and reactivity for each monoclonal antibody. Representative fluorescence images for the following vaccines on the VXI microarray: a) A/Vietnam/1203/2004 from H5 Clade 1 (egg derived, CBER Reference Antigen, Lot #50), d) A/Gryfalcon/Washington/41088-6/2014 from H5 Clade 2.3.4.4 (egg derived, IRR, Cat # FR-1447), e) A/Guangdong/17SF003/2016 from the H7N9 subtype (rHA), and f) A/chicken/Hong Kong/G9/1997 from H9 Clade G1 (NIBSC, Lot # 08/228).

important to note that this analysis was performed as a qualitative assessment of the coverage and specificity of each capture antibody within the VXI-pHA assay. Because the antigens differed in age and storage conditions, we cannot compare signal intensities between samples and make quantitative conclusions with any confidence since each antigen likely has a varying level of degradation.

The VXI-pHA assay detected 26/28 (93%) of the H5 antigens included in the panel with sufficient signal at assay relevant concentrations (Fig. 2). To assess the coverage of the VXI-pHA potency assay across the H5 subtype, a phylogenetic analysis was performed using the strain information for the 26 detected H5 antigens (Fig. 3a). The assay coverage broadly spanned the A/H5 phylogenetic tree, with confirmed detection of clades 1, 2, 4, and 7, which are the focus of candidate vaccine virus (CVV) development. CVVs are strains identified by the WHO for use in future vaccine development. The detection of all tested CVVs suggests that VXI-pHA would be able to quickly respond to the call for a new pandemic vaccine, which would likely be developed to one of these CVV strains. Additionally, the antigens detected represent 16 years of evolutionary time, from 1998 to 2014, suggesting the capture antibodies are probing fairly conserved
epitopes and would be less likely to be affected by antigenic changes in novel strains.

The two H5 strains not detected at signal intensity greater than 3× background were A/chicken/Vietnam/NCVD-03/2008-PR8-IDCDC-RG25A, an egg-based H5N1 antigen from the International Reagent Resource (IRR), and A/chicken/Netherlands/3295/2006, a recombinant H5N8 antigen from the Biodefense and Emerging Infections Research Resources Repository (BEI). In product literature for the rH5 antigen, it is mentioned that the antigen is not active in hemagglutination assays, suggesting that this antigen is conformationally defective. It is possible that the A/chicken/Vietnam/NCVD-03/2008-PR8-IDCDC-RG25A antigen was

| Strain Information | Relative Signal Intensity | Antigen Information |
|--------------------|--------------------------|---------------------|
| **Subtype**        | **Name**                 | **Clone**           | **H5(1)** | **H5(2)** | **H5(3)** | **H5(4)** | **H7(1)** | **H7(2)** | **H9(1)** | **H9(2)** | **H9(3)** | **Type** | **Source** | **Lot #** |
| H5                 | A/Cambodia/’X0910301/2013-PR8-IDCDC-RG34B | 1,1,1 | **Egg** | **IRR** | FR-1386 |
| H5                 | A/Vietnam/1203/2004       | 1     | | | |
| H5                 | A/Vietnam/1203/2004       | 1     | | | |
| H5                 | A/Vietnam/1203/2004       | 1     | | | |
| H5                 | A/Vietnam/1203/2004       | 1     | | | |
| H5                 | A/Vietnam/1203/2004-PR8-IDCDC-RG/50 | 1     | | | |
| H5                 | A/chicken/Yunnan/1215/2003 | 1     | | | |
| H5                 | A/duck/Hunan/795/2002     | 2.1,1 | **Egg** | **IRR** | FR-736 |
| H5                 | A/chicken/Indonesia/5/2005 | 2.1,2 | | | |
| H5                 | A/chicken/Indonesia/5/2005 | 2.1,3,2 | | | |
| H5                 | A/Egypt/3300-NAMRU3/2008-PR8-IDCDC-RG13 | 2.2,1,1 | | | |
| H5                 | A/Egypt/32/2007-PR8-IDCDC-RG11 | 2.2,1 | | | |
| H5                 | A/Egypt/32/2007-PR8-IDCDC-RG29 | 2.2,1 | | | |
| H5                 | A/bar-headed goose/Qinghai Lake1A/2005 | 2.2 | | | |
| H5                 | A/India/NIV/2006-PR8-IDCDC-RG7 | 2.2 | | | |
| H5                 | A/Hubei/1/2010-PR8-IDCDC-RG30 | 2.3,2,1 | | | |
| H5                 | A/duck/Laos/3295/2006     | 2.3,4 | | | |
| H5                 | A/common magpie/Hong Kong/645/2006 | 2.3,4 | | | |
| H5                 | A/Anhui/01/2005-PR8-IDCDC-RG6 | 2.3,4 | | | |
| H5                 | A/Anhui/01/2005           | 2.3,4 | | | |
| H5                 | A/Anhui/01/2005           | 2.3,4 | | | |
| H5                 | A/Anhui/01/2005           | 2.3,4 | | | |
| H5                 | A/Anhui/01/2005           | 2.3,4 | | | |
| H5                 | A/Pheasant/New Jersey/1355/1998-PR8-IDCDC-4 | 2.3,4 | | | |
| H5                 | A/Japanese white-eye/Hong Kong/1038/2006 | 2.3,4 | | | |
| H5                 | A/Guzhou/1/2013-PR8-IDCDC-RG35 | 2.3,4,2 | | | |
| H5                 | A/gyrfalcon/Washington/41088-6/2014 | 2.3,4,4 | | | |
| H5                 | A/gyrfalcon/Washington/41088-6/2014-PR8-IDCDC-RG43A | 2.3,4,4 | | | |
| H5                 | A/chicken/Netherlands/1401553/2014 | 2.3,4,4 | | | |
| H5                 | A/Sichuan/26221/2014-PR8-IDCDC-RG42A | 2.3,4,4 | | | |
| H5                 | A/chicken/Vietnam/NCDV-016/2008-PR8-IDCDC-RG12 | 7.1 | | | |
| H5                 | A/chicken/Vietnam/NCDV-03/2008-PR8-IDCDC-RG25A | 7.1 | | | |
| H7                 | A/Turkey/Virginia/2002-PR8-IDCDC-5 | H7N2 | | | |
| H7                 | A/Canada/444/2004         | H7N3 | | | |
| H7                 | A/Netherlands/219/2003    | H7N7 | | | |
| H7                 | A/Mallard/Netherlands/12/2000-PR8-IDCDC-1 | H7N7 | | | |
| H7                 | A/Shanghai/01/2013        | H7N9 | | | |
| H7                 | A/Shanghai/02/2013-PR8-IDCDC-RG32A | H7N9 | | | |
| H7                 | A/Shanghai/02/2013        | H7N9 | | | |
| H7                 | A/Shanghai/01/2013        | H7N9 | | | |
| H7                 | A/Shanghai/01/2013        | H7N9 | | | |
| H7                 | A/Shanghai/01/2013        | H7N9 | | | |
| H7                 | A/Anhui/01/2013           | H7N9 | | | |
| H7                 | A/Anhui/1/2013-NiBRG-268  | H7N9 | | | |
| H7                 | A/Anhui/01/2013           | H7N9 | | | |
| H7                 | A/Guangdong/17S/F003/2016 | H7N9 | | | |
| H7                 | A/Hong Kong/125/2017      | H7N9 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/chicken/Hong Kong/G9/1997 | Y280 | | | |
| H9                 | A/Hong Kong/33982/2009    | G1 | | | |
| H9                 | A/Hong Kong/1073/1999     | G1 | | | |
| H9                 | A/Hong Kong/33982/2009-PR8-IDCDC-RG26 | G1 | | | |
| H9                 | A/California/07/2009NYMC, (X-179A) | Y280 | | | |
| H1                 | A/Singapore/GP1908/2015 (IVR-180) | Y280 | | | |
| H3                 | A/Hong Kong/4801/2014 (X-263B) | Y280 | | | |
| B/V                | B/Brussels/60/2008       | Y280 | | | |
| B/V                | B/Phuket/3073/2013       | Y280 | | | |

**Fig. 2** Relative signal intensities for VXI-pHA capture antibodies against a panel of pandemic antigens. The listed panel of antigens were tested on VXI-pHA. White/empty boxes indicate signal intensity below the 3× background intensity cutoff, light green indicates signal intensity between 3x and 10x background intensity, green indicates between 10x and 20x background, dark green indicates between 20x and 40x background, and dark green with red asterisk indicates 40x to fluorescence saturation.
also compromised given that the assay did detect a similar reassortant, A/chicken/Vietnam/NCVD-016/2008-PR8-IDCDC-RG12.

The H7 capture antibodies detected 12 of the 13 H7 antigens tested (92%) within the antigen panel (Fig. 2). The H7 capture antibodies detected antigens spanning the phylogenetic tree for the H7 subtype (Fig. 3b), including the most recently emerged H7N9 strains as well as more distant A/H7 viruses dating back to 2000. All four of the CVVs tested were detected by the assay, including the latest A/Guangdong and A/Hong Kong CVVs that were recently announced.8 The one sample not detected by the assay was an H7N3 A/Canada/rv444/2004 recombinant HA sample. This sample is discussed further below.

Of the seven H9 antigens tested, the VXI-pHA assay detected seven (100%) (Fig. 2) with strains spanning two clades (Fig. 3c) and 12 years. While there were few antigens available for evaluation, likely due to less emphasis being put on H9 strains as potential pandemic strains, the two VXI-pHA capture antibodies demonstrated good coverage.

To assess the compatibility of the VXI-pHA assay with vaccines produced using different production platforms, we screened antigens derived from egg-based, cell-based, and recombinant technologies. The VXI-pHA assay detected 86% (6/7), 83% (5/6), and 100% (3/3) of the non-egg-derived H5, H7, and H9 antigens, respectively. All five H5 capture antibodies were capable of detecting non-egg-derived samples. As an example of direct comparison, two A/Indonesia/05/2005 antigens, one recombinant from a baculovirus system and one egg-propagated, each demonstrate high signal intensities on antibodies H5(i), H5(ii), and H5(iii) (Fig. 2). The one non-egg-derived H7 antigen that was not detected was an A/Canada/rv444/2004 recombinant

Fig. 3 VXI-pHA coverage across the H5, H7, and H9 subtypes. Phylogenetic trees of potentially pandemic vaccine-relevant strains of A/H5 a, A/H7 b, and A/H9 c influenza. Candidate vaccine virus strains (CVVs) are distinguished by a ◆. Strains that have been tested on the VXI-pHA assay and have been positively identified are marked by red text. Strains in black have not yet been tested.
antigen. Because there was only a single H7N3 antigen available for testing, it is not clear if the assay is not compatible with H7N3 antigens in general, recombinant H7N3 antigens, or if there was an issue with antigen stability.

The data presented in Fig. 2 were used to evaluate each capture mAb for specificity, which was defined as the number of subtype-specific detection events (signal over 3× background) divided by the total number of detection events for that antibody. For this...
study, seasonal HA antigens were included to determine whether or not the pandemic capture mAbs had any cross-reactivity with seasonal antigens. Four of the five H5 capture mAbs detected only H5 and did not detect H7, H9, H1, H3, or influenza B HA antigens tested, demonstrating 100% specificity to H5 antigen. The H5(iii) capture mAb detected one whole virus H7N9 A/Shanghai/1/2013 antigen (Fig. 2) at 3–10× background signal, resulting in a corresponding specificity of 95%. All other VXI-pHA capture mAbs, H7(i–ii) and H9(i–ii), demonstrated 100% specificity with no cross-reactivity with other pandemic or seasonal subtypes.

The linear dynamic range of VXI-sHA is more than 150-fold

Thirteen-point serial dilutions of H5, H7, and H9 reference antigens with known HA concentrations were tested by the VXI-pHA assay over a range of 0.001–1.25 µg/mL to evaluate each capture mAb for linear dynamic range (LDR) as well as upper and lower limits of quantification. All of the VXI-pHA capture antibodies produced robust, linear curves in response to increasing antigen concentrations (Fig. 4a–d). The H5(ii) antibody exhibited higher sensitivity (i.e., steeper slope) to the A/Indonesia/05/2005 antigen than the H5(i) and H5(iii) capture mAbs (Fig. 4a). Similarly, the H7(ii) antibody exhibited higher sensitivity to the A/Shanghai/02/2013 antigen than the H7(i) antibody (Fig. 4c). This is not unexpected as different antibodies often have very different binding constants. Because the assay utilizes a calibration curve, the differences in antibody binding constants does not result in different concentrations determined by each antibody. Using each calibration curve, LDRs were calculated and reported in Table 1. The LDRs are ≥150-fold for all capture antibodies and are at least 15 times greater than the quantification range of SRID (~6–30 µg/mL).

VXI-pHA is a more sensitive potency assay than SRID

Three of the four antigens (A/H5 A/Indonesia, A/H7 A/Shanghai, and A/H9 A/chicken/HK) used to assess the LDR of VXI-pHA were analyzed by SRID with appropriately matched reference antisera to provide a side-by-side comparison of the sensitivity of the two assays. The A/H5 A/gryrfalcon sample was not analyzed by SRID because at this time there are no SRID reference reagents available. Eight antigen concentrations were tested, from 0.5 to 40 µg/mL of HA, to test the lower and upper limits of the SRID assay. A representative SRID response curve for the H7 antigen is presented in Fig. 4e, f. For each antigen tested, the LDR of the assay was determined using a previously reported method.9 Briefly, a linear regression was fit to the points corresponding to the reported LDR for SRID9 (red points in Fig. 4e). Next, the measured diameter for each concentration tested was compared to the expected diameter based on the linear regression using a one sided, one-sample t-test (p < 0.05) to determine if any given data point significantly deviated from the regression. Concentrations that significantly deviated from the regression were considered outside the LDR of the assay. Using this method, the LDRs for H5, H7, and H9 SRID assays were calculated and are presented in Table 1. For H5 and H7, the reported LDR of the SRID assay remained 5–30 µg/mL and was not expanded. For H9, the 40 µg/mL concentration did not significantly deviate from the linear regression, and thus the upper limit of quantification for H9 was determined to be ≥40 µg/mL. Concentrations higher than 28 µg/mL of reference antigen A/H5 A/Indonesia were not tested due to a low initial antigen concentration (32 µg/mL).

The LDR for each of the VaxArray capture antibodies was compared to the measured LDRs for SRID (Table 1). For H5, the SRID LDR was 5.4x whereas the VaxArray LDRs were 300x, 500x, and 500x for the H5(i), H5(ii), and H5 (iii) antibodies, respectively. For H7, the SRID LDR was 5.8x whereas the VaxArray LDRs were 375x and 750x for H7(i) and H7(ii) antibodies, respectively. For H9, we could not calculate an SRID LDR because we did not define an upper limit of the assay. In further comparing the two assays, the average lower quantification limit for VaxArray was 2650-fold lower than for SRID (0.002±0.001µg/mL compared to 5.3±0.1µg/mL for SRID). The lower limits of quantification greatly reduce the amount of sample required compared to SRID, a crucial benefit for quantification of dose-sparing vaccines and/or vaccines in high demand. Additionally, the large dilution factors allowed by this low limit of quantification can greatly dilute out interfering substances such as crude matrix proteins and adjuvants, all known inhibitors of the SRID assay.5

VXI-pHA demonstrates a high level of accuracy

To assess the performance of the VXI-pHA assay, four different production lots of an A/Hong Kong/125/2017 H7N9 monobulk drug substance were evaluated by VXI-pHA following the standard procedure. At the time of this study, SRID reference reagents were not available for this new “5th wave” H7 influenza vaccine in development and could not be used as a reference standard. To overcome this hurdle, the purity-adjusted bicinchoninic acid (paBCA) assay was used to determine total HA protein content of one of the production lots. This sample was determined to have 154±17 µg/mL HA and was then used as an internal standard to calibrate the VXI-pHA assay. This approach is not unreasonable, since a purity-adjusted total protein measurement is used to define the HA concentration within the Primary Liquid Standard used by WHO laboratories to calibrate reference reagents.10 Using the internal standard for calibration, three other production lots of the monobulk drug substance were analyzed by the VXI-pHA assay using both H7 antibodies (i and ii) in replicates of seven on three separate days. The same three production lots were also analyzed on three separate days in replicates of three for HA content by the paBCA method. The accuracy of the VXI-pHA measurements was assessed by comparing the concentrations determined by VXI-pHA to those determined by paBCA and was defined as “percent of paBCA”. By comparing VXI-pHA to paBCA, the assumption must be made that the protein content of the monobulk is 100% intact as the paBCA method is a total protein measurement and not able to distinguish between intact and degraded protein. The percent agreement between methods for the three monobulk production lots is 105%, 104%, and 106% for the H7(ii) VXI-pHA capture antibody and 103%, 101%, and 107% for the H7(ii) VXI-pHA capture antibody (Fig. 5a). Note the H7(ii) VXI-pHA capture antibody performed very similarly and within error of H7(i) demonstrating that either antibody can be used to quantify H7 antigen.

### Table 1. Quantification ranges for VXI-pHA and SRID

| Subtype | mAb ID | Lower QL (µg/mL) | Upper QL (µg/mL) | Range |
|---------|--------|-----------------|-----------------|-------|
| H5      | H5(i)  | 0.003           | 1               | 300x  |
|         | H5(ii) | 0.001           | 0.5             | 500x  |
|         | H5(iii)| 0.002           | 1               | 500x  |
|         | H5(iv) | 0.001           | 0.5             | 500x  |
|         | H5(v)  | 0.001           | 0.5             | 500x  |
|         | SRID   | 5.3             | 28.7            | 5.4x  |
| H7      | H7(i)  | 0.002           | 0.75            | 375x  |
|         | H7(ii) | 0.001           | 0.75            | 750x  |
|         | SRID   | 5.4             | 31.3            | 5.8x  |
| H9      | H9(i)  | 0.005           | 0.75            | 150x  |
|         | H9(ii) | 0.004           | 0.75            | 187x  |
|         | SRID   | 5.3             | >40.6           | >7.7x |
VXI-pHA demonstrates a high level of precision
The data generated in the accuracy study also included an assessment of precision since three production lots of the monobulk drug substance were analyzed by VXI-pHA in replicates of seven on three separate days. In each analysis, an 8-point calibration curve was run. The calibration curves generated on each day were compared (Fig. 5b) and the slope and associated error were found to be within error of one another demonstrating the high reproducibility of the assay. The replicate analysis resulted in the following HA concentrations of the three monobulk formulations: $185 \pm 15$, $186 \pm 10$, $182 \pm 9 \mu g/mL$ (Fig. 5c). The intraday assay precision was 5%, 5%, and 7% for day one, two, and three, respectively (Table 2). The average error across all replicates tested was 6% for the three samples. For comparison, the average relative error reported for the SRID assay is 12%; thus, VXI-pHA offers improved precision for the quantification of potency.
VXI-pHA is capable of detecting changes in protein conformation. Vaccine manufacturers are required to monitor the potency of their vaccines at batch release and over time after release. Additionally, due to the need to release pandemic vaccines as quickly as possible, manufacturers often perform forced degradation studies to predict vaccine stability before batch release. To test the stability indication properties of the VXI-pHA assay, we performed a representative forced degradation experiment for a panel of antigens designed to address the stability indication capabilities of each capture antibody. Briefly, samples were analyzed in triplicate by VXI-pHA and SRID before (T0) and after a 20 h incubation at 56 °C (T20) and %T0 values were calculated. Results are presented as %T0 (T20/T0) for each capture antibody (see legend). Antigens used are shown on the x-axis. e VXI-pHA potency determination of solutions containing HA antigens spiked into allantoic fluid, 40% sucrose, and used DMEM medium from uninfected MDCK cells. The following antigens were used: A/Indonesia/05/2005 CBER Lot #83 (H5N1), A/gyrfalcon/Washington/41088-21/2014, ~0% for H7 A/Shanghai/02/2013, and <8.7% for H9 A/gyrfalcon/Washington/41088-21/2014, <0.3% for H5 A/gyrfalcon/Washington/41088-2014, ~0% for H7 A/gyrfalcon/Washington/41088-21/2014, and <8.7% for H9 A/chicken/Hong Kong/G9/1997 (H9N2). e VXI-pHA potency determination for antigens spiked into adjuvants. Antigens were mixed with 1.7 mg/mL elemental aluminum or 19.5 mg/mL MF59 for a final HA concentration of 2.5 µg/mL before being diluted and analyzed by VXI-pHA. For each sample, a PBS negative control was included. Antigens included A/Indonesia/05/2005 CBER Lot #83 (H5N1), A/Shanghai/02/2013 vaccine (H7N9), and A/chicken/Hong Kong/G9/1997 Lot # U51P72H1 from BEI (H9N2). For both e and f, each data point represents a single replicate. The thick black bars represent the average across the four replicates. Error bars represent the standard deviation across the four replicates for each sample. The red-dotted line represents the expected concentration for each sample. The shaded red region represents the expected concentration plus and minus the error associated with the assay.
A non-adjuvanted negative control sample was included where phosphate-buffered saline (PBS) was added instead of adjuvant. Monovalent mock-formulated dose-sparing vaccines were generated for the following strains: A/Indonesia/05/2005 (H5N1), A/Shanghai/02/2013 (H7N9), and A/chicken/Hong Kong/G9/1997 (H9N2). Each monovalent mock-formulated vaccine and negative PBS control was properly diluted to within the LDR of the VXI-pHA assay and analyzed against a corresponding, non-adjuvanted 8-point calibration curve. The expected concentration for all samples was 5.0 µg/mL (demonstrated by the red-dotted line in Fig. 5f). All samples, despite subtype and adjuvant-type, were measured on average to be within 10% of the expected concentration (red shading in Fig. 5f). The differences between samples is likely due to some noise introduced during sample preparation (i.e., pipetting) and is not due to the presence of adjuvant as the noise is also evident in the PBS, non-adjuvanted sample. These findings suggest that the VXI-pHA assay is a reliable measure of potency (±10%) even in the presence of aluminum hydroxide- or squalene-based adjuvants.

**DISCUSSION**

The development of vaccines against potentially pandemic influenza strains is a critical piece in the global response to an influenza pandemic. Immediate access to a potency assay for monitoring of immunogenic HA concentrations would streamline the development, production, release, and ongoing stability monitoring of pandemic vaccines, such as those stored in the National Influenza Vaccine Stockpile. To address the current bottleneck in influenza vaccine potency determination, an on-demand potency assay was developed for influenza vaccines produced in response to the most concerning influenza A subtypes. Specifically, new strains of avian influenza H5 and H7 subtypes continue to infect humans who work closely with poultry, which has led the WHO to recommend the development of new CVVs to address divergence from older CVVs.

The VXI-pHA assay relies on a panel of monoclonal antibodies designed to cumulatively detect HA from H5, H7, and H9 strains spanning several years despite antigenic drift in the influenza HA protein. Overall, the panel of anti-H5, H7, and H9 mAbs selected for inclusion in the VXI-pHA assay exhibits good specificity, high sensitivity, and broad coverage over a large span of viral evolutionary time including nearly all the proposed CVVs.

Interestingly, HA from recently isolated A/H7N9, which resulted in the recently announced A/Guangdong and A/Hong Kong CVVs, are detected by the same antibodies that detect the much more distant A/H7N7 and A/H7N2 strains of influenza, indicating binding to a relatively conserved epitope. The robust responses for each subtype offers a measure of confidence that the test would be viable over some degree of HA protein evolution.

While the VXI-pHA assay has 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 VXI-pHA assay is not able to detect. In the event that a new strain is not detected on the current version of the array, InDevR has developed a protocol to rapidly screen all available mAbs in a highly multiplexed format. If an mAb exists that can detect the new strain, it can be incorporated into the array, verified, and validated in an expedient, but quality managed, process. If no mAb exists, InDevR would leverage close collaboration with a number of government agencies to develop an appropriate mAb on an accelerated time scale. As a fall-back position for emergency use, we are also investigating the possibility of incorporating “universal” mAbs for detection and quantification.

While these mAbs do not offer subtype specificity, that level of specificity may not be needed for a monovalent pandemic vaccine.

The VXI-pHA assay also has broad coverage across different vaccine production methods. Historically, influenza vaccines have been mass produced in embryonated chicken eggs, but recent developments in cell-culture based and recombinant protein production platforms have broadened the potential sources of vaccine antigens. Data reported here demonstrate that the VXI-pHA assay is capable of being used to determine the potency of novel, recombinant, and cell-based vaccines in addition to traditional, egg-based vaccines, thus overcoming the incompatibility issues that manufacturers of novel vaccines such as rHA vaccines and virus-like particle (VLP) vaccines have faced with SRID.

Many of the benefits of the VXI-pHA assay are due to the broad LDR of the assay which is at least 15 times greater than the quantification range in SRID (~6–30 µg/mL). Additionally, the lower limit of quantification for the assay greatly reduces the amount of sample required compared to SRID, a crucial benefit for quantification of dose-sparing vaccines and/or vaccines in high demand. Additionally, the large dilution factors allowed by this low limit of quantification can greatly dilute out interfering substances such as crude matrix proteins and adjuvants, all known inhibitors of the SRID assay. Common adjuvants such as aluminum hydroxide and squalene-based adjuvants like MF59 are used to deliver antigen dose-sparing vaccines while maintaining the efficacy of larger doses, thereby enabling broader protection of the population. Due to the inherent incompatibility of SRID with some adjuvants, as well as concern over the stability of antigen in the presence of adjuvants over time, most adjuvanted vaccines are currently stored separately from adjuvants in two vials and mixed bed-side before administration to patients. Because the VXI-pHA can be applied to low-dose adjuvanted vaccines, it could be utilized to monitor potency in the presence of adjuvants. With more information at hand on the stability and potency of vaccines stored in adjuvants for extended periods of time, the need for the two-vial system could be reassessed.

The major time benefit of VXI-pHA over SRID is that the assay is not reliant on the time-consuming production process for generating reference reagents for calibration of the assay. Our approach to calibrating the VXI-pHA assay in this study was to utilize an internal standard by quantifying total HA using the paBCA method. In the case of a pandemic, it is likely that vaccine manufacturers would take a similar approach for tracking HA concentration during vaccine development.

There is increasing scientific evidence that neuraminidase (NA) within influenza vaccines leads to NA immunity, decreased viral shedding, and reduced severity of influenza disease. For this reason, we have developed a VaxArray assay for the quantification of NA in seasonal influenza vaccines. The assay utilizes the same technology as VXI-pHA, which is made modular by simply changing the capture and label antibodies for NA-specific reagents. Future efforts will expand the technology to include an assay for pandemic NA, allowing manufacturers to track both HA and NA in pandemic vaccines in rapid fashion.

In summary, the VXI-pHA assay is reagent sparing, stability indicating, and capable of accurately and precisely monitoring vaccine potency of low-dose, adjuvanted pandemic vaccines. The VXI-pHA assay is also capable of monitoring HA concentration in crude in-process samples, potentially allowing manufacturers to increase vaccine yield, and ultimately the amount of life-saving vaccines they are able to produce.

**METHODS**

**VXI-pHA standard procedure**

The VXI-pHA technology is similar to the VaxArray Influenza Seasonal Hemagglutinin Potency Assay (VXI-SHA) described previously. VXI-pHA reagent kits (Cat# VXI-7200; InDevR Inc.) contain two microarray slides,
printed with 16 replicate arrays per slide, Fiducial Detection Label, Protein Blocking Buffer (PBB), and two Wash Buffers. Prior to use, VXi-pHA slides were removed from the refrigerator and equilibrated to room temperature for 30 min in the provided foil pouch. Samples were prepared individually by lysing at room temperature for 30 min in the presence of 1% 

with an acceptable linear fit ($R^2 > 0.95$). The lower limit of quantification for each antibody, defined by the median signal of the capture antibody spots in the antigen blank plus five times the standard deviation across these spots, was calculated individually for each capture antibody with each antigen blank and then averaged.

Stability indication studies
Antigen samples were diluted in PBS to a final concentration of 15 µg/mL HA and 100 µL aliquots were added to 1.5 µL amber glass vials, sealed with a crimp top, and weighed. 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 reweighed before analysis to check for possible evaporation during degradation. All weights showed <0.07% difference after degradation. Samples before (T0) and after 20 h at 56 °C (T20) were analyzed in triplicate with an 8-point standard curve of non-degraded antigen using the VXi-pHA potency assay using the standard procedure described above.

Quantification of HA in crude matrix
HA-containing antigens were spiked into a 40% sucrose solution (Cat# S9378; SigmaAldrich), allantoic fluid from 10-day-old embryonated chicken eggs (Cat# BV027; Virapuri), and exhausted DMEM + 10% fetal bovine serum tissue culture media taken from non-infected MDCK cells (provided by a collaborator) to mock 2.5 µg/mL HA solutions in each matrix. All spiked samples were lysed with 1% Zwittergent 3-14 for 30 min and diluted in PBB/Z to a final expected concentration of 0.1 µg/mL HA and analyzed by VXi-pHA in quadruplicate ($n = 4$) using the standard procedure described above. A standard curve of antigen was prepared by lysing the same antigens in PBS and 1% Zwittergent, serially diluting in PBB/Z, and was analyzed alongside the crude-matrix spike antigens by VXi-pHA. The HA concentration in each spiked sample was determined against the appropriate calibration curve. The average HA concentration determined for each spiked sample was compared to the expected sample concentration based on the known concentration of the antigen and the performed dilutions. The percent difference between the expected and measured HA concentrations was calculated for each sample.

Quantification of HA in mock dose-sparing, adjuvanted vaccines
Three HA-containing antigens were individually spiked into aluminum hydroxide (Cat# 77161; ThermoFisher), MF59 (Cat# Vac-adx-10; Invivogen), and PBS at final concentrations of 5.0 µg/mL of HA resulting in two “mock” adjuvanted vaccines with adjuvant concentrations of 0.17 mg/mL of elemental aluminum or 1.95 mg/mL squalene and a negative PBS control. Each solution was incubated at room temperature for 30 min in adjuvant or PBS before being lysed with 1% Zwittergent for 30 min. The “mock” adjuvanted vaccines and the corresponding negative controls were diluted in PBB/Z to 0.1 µg/mL for the H5 A/Indonesia sample (CBER, Lot #83) and 0.25 µg/mL HA for the H7 A/Shanghai (CBER, Lot #78) and H9 A/chicken/Hong Kong (Cat# NR-12140, BEI) sample and analyzed by VXi-pHA in quadruplicate ($n = 4$) against an eight-point calibration curve of a matched non-adjuvanted antigen. Using the standard VaxArray procedure, average HA concentrations were determined for each “mock” adjuvanted vaccine and negative control using the appropriate standard curve. The HA concentrations of each sample were compared to the expected HA concentration, based upon the known dilution factors, to investigate the effect of aluminum hydroxide and MF59 on VXi-pHA performance compared to the negative control.

Single radial immunodiffusion
SRID was performed as described previously with minor deviations. Three separate agarose preparations were prepared by dissolving 1.1 g of agarose (Cat# 50011; Lonza) in 100 mL of PBS and kept at 55 °C. Antiserum solutions for H5 A/Indonesia (CBER, Lot# H5-As-1217), H7 A/Shanghai (CBER, Lot# H7-Ab-1402), and H9 A/chicken/Hong Kong (NIBSC, Lot# 08/202) were reconstituted in ultrapure water according to their associated instructions and added to the agarose solutions. For each subtype, the agarose solution was swirled to mix and 25 µL was added to gelbond film (Cat# 53734; Lonza) and allowed to cool. After solidifying, 25 4-mm holes were punched into each gel using a biopsy punch (Cat# 96–1115; Sklar Instruments). Each sample to be evaluated was lysed in PBS and 1%...
Purity-adjusted total protein by bicinchoninic acid assay
For each H7N9 monovalent drug substance, the total protein content was determined by the microbicinchoninic acid (BCA) assay (Cat# 23235; ThermoFisher). Next, 20 µL of each sample were denatured and reduced at 95 °C for 5 min followed by deglycosylation in the presence of PNGase F (Cat# V4831; Promega) overnight at 37 °C. The XCell SureLock gel box and NuPAGE® pre-cast 4–12% bis-tris gradient gels (Cat# NP0322; ThermoFisher) were used to evaluate each deglycosylated monobulk sample alongside a non-treated monobulk to evaluate band shifts. Gels were stained with Coomassie Brilliant Blue R250 (Cat# 57055; ThermoFisher). Next, 20 µL of each sample were denatured and reduced at 95 °C for 5 min followed by deglycosylation in the presence of PNGase F (Cat# V4831; Promega) overnight at 37 °C. The XCell SureLock gel box and NuPAGE® pre-cast 4–12% bis-tris gradient gels (Cat# NP0322; ThermoFisher) were used to evaluate each deglycosylated monobulk sample alongside a non-treated monobulk to evaluate band shifts. Gels were stained with Coomassie Brilliant Blue R250 (Cat# 57055; ThermoFisher).

Code availability
The mathematically algorithm used to analyze the VXI-pHA assay can be accessed in a previously published study.7

DATA AVAILABILITY
All relevant data from this study are available from the authors.

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AUTHOR CONTRIBUTIONS
R.T.B.-N. and D.F.M. were responsible for experimental design. R.T.B.-N. prepared the manuscript. J.H.G. and K.M.B. executed many of the experiments. K.L.R. invented the assay and performed the statistical analysis. R.T.B.-N. and D.F.M. were responsible for experimental design. R.T.B.-N. prepared the manuscript. J.H.G. and K.M.B. executed many of the experiments. K.L.R. invented the assay and performed the statistical analysis.

ADDITIONAL INFORMATION
Competing interests: K.L.R. and L.R.K. are InDevR Inc. stockholders. The remaining authors are employed by InDevR Inc. but declare no competing interests.

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REFERENCES
1. Schneisser, F., Vodeiko, G. M., Lugo-Servaites, Y. V., Stout, R. R. & Wei, J. P. An alternative method for preparation of pandemic influenza strain-specific antibody for vaccine potency determination. Vaccine 28, 2442–2449 (2010).
2. Li, C. et al. Application of deglycosylation and electrophoresis to the quantification of influenza viral hemagglutinins facilitating the production of 2009 pandemic influenza (H1N1) vaccines at multiple manufacturing sites in China. Biologicals 38, 284–289 (2010).
3. Vodeiko, G. M. & Wei, J. P. Determination of H5N1 vaccine potency using reference antisera from heterologous strains of influenza. Influenza Other Respir. Viruses 6, 176–187 (2012).
4. World Health Organization. WHO Guidelines on the use of vaccines and antivirals during influenza pandemics: WHO/CDS/CSR/RMD/2004.8. http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_RMD_2004_8/en/index.html (2004).
5. Minor, P. D. Assaying the potency of influenza vaccines. Vaccines 3, 90–104 (2015).
6. Kuck, L. R. et al. VaxArray assessment of influenza split vaccine potency and stability. Vaccine 35, 1918–1925 (2017).
7. Kuck, L. R. et al. Titer on chip: new analytical tool for influenza vaccine potency determination. PLoS ONE 9, e109616 (2014).
8. World Health Organization. Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. 15. http://www.who.int/influenza/vaccines/virus/201709_zoonotic_vaccinevirusupdate.pdf?ua=1 (2017).
9. Mancini, G., Carbonara, A. O., & Heremans, J. F. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2, 235–254 (1965).
10. 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. http://www.who.int/medicalproducts/areas/vaccines/TRS_979_Annex_5.pdf (2013).
11. Yen, C. et al. The development of global vaccine stockpiles. Lancet Infect. Dis. 15, 340–347 (2015).
12. Li, C. et al. A simple slot blot for the detection of virtually all subtypes of the influenza A viral hemagglutinins using universal antibodies targeting the fusion peptide. Nat. Protoc. 5, 14–19 (2010).
13. Thompson, C. M., Peliot, E., Lenaertsz, A., Henry, O. & Kamen, A. A. Analytical technologies for influenza virus-like particle candidate vaccines: challenges and emerging approaches. Viral. J. 10, 141 (2013).
14. Marcelin, G., Sandbulte, M. R. & Webby, R. J. Contribution of antibody production against neuraminidase to the protection afforded by influenza vaccines. Rev. Med. Virol. 22, 267–279 (2012).
15. Kilbourne, E. D. Comparative efficacy of neuraminidase-specific and conventional influenza virus vaccines in induction of antibody to neuraminidase in humans. J. Infect. Dis. 134, 384–394 (1976).
16. Kilbourne, E. D., Laver, W. G., Schulman, J. L. & Webster, R. G. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J. Virol. 2, 281–288 (1968).
17. Memoli, M. J. et al. Evaluation of antihemagglutinin and antineuraminidase Antibodies as correlates of protection in an Influenza A/H1N1 Virus Healthy Human Challenge Model. mbio 7, e00417–16 (2016).
18. Eichelberger, S. L. et al. Potency under pressure: the impact of hydrostatic pressure on antigenic properties of influenza virus hemagglutinin. Influenza Other Respir. Viruses 7, 961–968 (2013).