VaxArray® Influenza Seasonal Hemagglutinin Potency Assay: Best Practice to Determine Accurate Concentration

Background: The VaxArray Seasonal Hemagglutinin Potency Assay is a new tool for hemagglutinin (HA) protein quantification based on a panel of subtype-specific but broadly reactive monoclonal antibodies (mAbs). Multiple antibodies against seasonal A/H1, A/H3, B/Yamagata-like and B/Victoria-like strains are printed in an array format on a glass substrate. As a multiplexed immunoassay, signal readout is based on fluorescence from a conjugated "universal" primary antibody label.

The VaxArray Influenza relies on a serially diluted calibration curve as well as accurate sample dilution factors to determine the HA concentration of samples. This assay is able to measure HA at very low concentrations and because of this high level of sensitivity inaccuracies inherent in micropipettes are magnified.

Objective: The objective of this technical note is to outline the determination of accurate concentrations by using mass (rather than volume) when the most accurate potency determination is required.

Although calibrated micropipettes are always used in the lab, a laboratory balance can make highly sensitive measurements which translate to a more precise and accurate quantitation of volume as the uncertainty of a micropipette is much higher than that of a balance. For example, an adjustable volume pipette that can dispense 2 to 200 µL can have an accuracy of ± 0.5 to 1.6 µL. An analytical balance has an accuracy of ± 0.1 mg, which translates to 0.1 µL of water. The uncertainty of a micropipette can be further compounded when multiple pipetting events are required as in a serial dilution series.

During VaxArray Influenza testing performed at InDevR, it was found that the concentrations and dilution factors determined using mass measurements can differ from the value determined using volume measurements by more than ten percent. For example, a dilution series that should result in a 1:800 dilution by volume (20 µL diluted to 600 µL (1:30), followed by 20 µL diluted to 533 µL) yielded an actual dilution factor of 1:861 when evaluated using measured mass. For a serially diluted calibration curve, the error can be further amplified as demonstrated in **Figure 1**.

Figure 1 – Comparing Concentration Determined Using Volume vs. Mass Measurements

Dilution #	Concentration (µg/mL)		% Difference
	By Volume	By Mass	
1	3.2	3.02	-5.6%
2	1.6	1.49	-6.9%
3	0.8	0.736	-8.0%
4	0.4	0.364	-9.0%
5	0.2	0.181	-9.5%
6	0.1	0.089	-11.0%
7	0.05	0.044	-12.0%
8	Blank	Blank	N/A

The reagents that are used in the VaxArray Influenza, as well as most standards and samples,

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have densities that are the same as water (1 g/mL), within reasonable error. It is therefore possible to arrive at measurements of volume (and hence dilution factor and standard concentration) from the mass measured by an accurate and precise balance. Samples or standards that have an unknown density (such as sucrose gradient purified materials) may not be quantifiable by this method.

Recommended Procedure: When preparing standards and samples, the concentration of standards and dilution factors of samples can be determined using mass measurements as follows.

For initial dilution of calibration standards during lysis with detergent:

1. Weigh the vial (**V**).
2. Using a pipette, add dilution buffer (typically PBS) and detergent (typically Zwittergent) to the vial and weigh the vial + buffer + detergent (**VBD**).
3. Using a pipette, add the stock standard to the vial and weigh the vial + buffer + detergent + standard (**VBDS**).
4. The stock concentration (µg/mL) of the standard will be designated as $\mathbf{C_1}$.
5. Calculate the final concentration, $\mathbf{C_2}$ (µg/mL), using the following equation:

$$\mathbf{C_2} = \frac{\mathbf{C_1} * (\mathbf{VBDS} - \mathbf{VBD})}{(\mathbf{VBDS} - \mathbf{V})}$$

For samples, the same measurements will be taken and the dilution factor (**DF**) will be calculated as follows:

$$\mathbf{DF} = \frac{(\mathbf{VBDS} - \mathbf{V})}{(\mathbf{VBDS} - \mathbf{VBD})}$$

Continue using the balance for further dilutions of standards and samples. For these second dilutions, the buffer used must be Protein Blocking Buffer. If possible, standards and samples should be diluted at least 1:1 (1:10 for crude samples) in Protein Blocking Buffer in order to reduce non-specific binding on the arrays. In some cases the Protein Blocking Buffer can be added directly to the vials containing the lysed sample and standard, followed by reweighing to determine final dilution factors and standard concentration.

Summary: Determining standard concentrations and sample dilution factors using mass measurements instead of relying on volume will yield far more accurate HA concentration results from the VaxArray Influenza.