VaxArray Data Extraction and Analysis Tech Note

Overview. ICH guidelines (Q2R1) indicate that *"Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content."* Furthermore the guidelines state: *"For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified."*
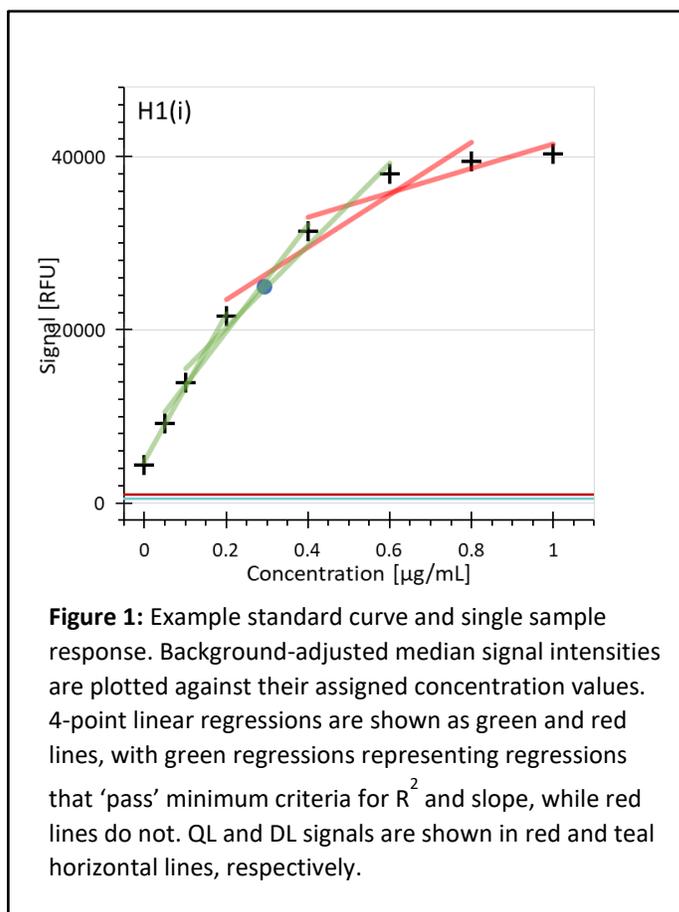
For establishment of VaxArray linearity, an 8 point standard curve is recommended for sample quantification. As with all immuno-sandwich assays, over a sufficiently large concentration range the trend may exhibit a plateau region where either binding or signal is saturated. A representative standard curve that illustrates this behavior is shown in **Figure 1.** Rather than fitting the data to a typical 4 parameter logistic function often employed for ELISA, which can lead to significant error in measured concentrations within or near the plateau region, the VaxArray Analysis Software utilizes 5 adjacent 4-point linear regressions – as illustrated by the solid lines in **Fig.1**. Samples whose fluorescence signals fall within one or more of the linear regression regions (delineated with + symbols in **Fig. 1**) are quantified using the appropriate calibration variables.

In this technical note, we describe the details behind data extraction and processing, from raw data to final result, and consistency with ICH guidelines.

Step 1: Extraction of Raw Data from Fluorescence Image.

The VaxArray Imaging System utilizes a sensitive camera to capture fluorescence images of each array on the slide. An example image is shown in **Figure 2**. The VaxArray Analysis Software employs a sophisticated spot finding algorithm to identify each fluorescence
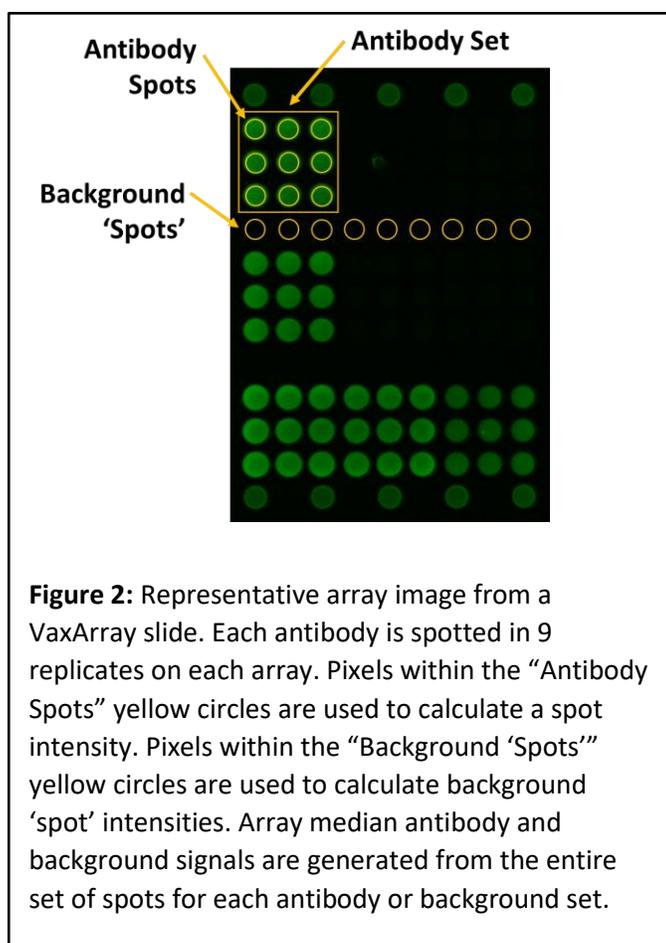
spot that represents the signal from each location where antibodies have been printed. Each spot is ~200 µm in diameter, which translates into ~500 pixels. The median signal intensity from all of the pixels within the identified spot is calculated and used as the signal for that spot.

Figure 1: Example standard curve and single sample response. Background-adjusted median signal intensities are plotted against their assigned concentration values. 4-point linear regressions are shown as green and red lines, with green regressions representing regressions that 'pass' minimum criteria for R^2 and slope, while red lines do not. QL and DL signals are shown in red and teal horizontal lines, respectively.

Step 2: Determination of Signal for Each mAb Set.
Each antibody is printed in 9 replicates, which are designated as "antibody set" in **Figure 2**. For a given antibody set, the median signal the nine spots is calculated . This approach ensures that outliers, such as a dust specks and other artifacts, do not unduly affect the signal intensity.

Step 3: Determination of Background. As with capture antibody spots, "background spots" are identified and their median signal intensity extracted. **Fig. 2** illustrates the space between antibody sets, where no fluorescent signal is expected, as an example of "background spots". These "spots" are processed in the same way described for each antibody set. For a given array, 20 such background spots are examined. The overall array background is calculated by calculating the median of these spots.

Figure 2: Representative array image from a VaxArray slide. Each antibody is spotted in 9 replicates on each array. Pixels within the "Antibody Spots" yellow circles are used to calculate a spot intensity. Pixels within the "Background 'Spots'" yellow circles are used to calculate background 'spot' intensities. Array median antibody and background signals are generated from the entire set of spots for each antibody or background set.

Step 4: Background Corrected Signal.

The above calculation yields the median intensity of the antibody set and of the array background. Factors other than analyte detection contribute to the fluorescent

intensity of these sets. These effects are measured and accounted for in the array background measurement. To accurately measure analyte-detection response, the VaxArray Analysis Software subtracts the array background from each antibody set's median signal intensity, generating a "background corrected signal" for each antibody in each array. These background corrected signals are used for all further analysis.

Step 5: Determination of Quantitation Limit. As the lowest point in the 8-point standard curve, a blank, or negative control is required. This approach allows for rapid evaluation of the quantitation limit (QL) for all antibody sets in this highly multiplexed assay. Within the blank well, the median signal of each antibody set is determined as described in steps 2 and 3. In addition, the standard deviation in signal is determined after rejecting the highest and lowest signal values as a measure of noise. This approach ensures that outliers, such as a dust specks and other artifacts, do not unduly affect the blank signal intensity standard deviation. The quantitation limit for a given antibody is defined as the median signal of the antibody set in the blank array plus a user-adjustable multiplier of the standard deviation in the signal.

Note that this calculation utilizes signal from the antibody spots in the blank well and *NOT* an array background. The blank array is never incubated with antigen, only Protein Blocking Buffer and label solution. By utilizing this array for calculating the quantitation limit, we are evaluating the fluorescent noise of the assay as a whole. The array background is used to evaluate and correct for fluorescence due to matrix, autofluorescence, and non-specific labeling in each array individually.

Step 6: Regression to Standard Curve.

As with all quantitative assays, VaxArray relies on a serial dilution of a known standard to enable quantification of analyte in an unknown. The standard curve is generated by plotting the 8 background-adjusted median signals of the antibody against the user-defined standard concentrations (see **Fig. 1**).

The VaxArray Analysis Software evaluates each curve with 5 adjacent 4-point ranges, as illustrated in **Figure 3.** Each 4-point range is fit using a linear regression and the quality of fit is evaluated through the Pearson's correlation coefficient. The user can specify the required quality of fit (e.g., $R^2 > 0.95$) and the minimum acceptable slope. If a given regression range does not meet the specified criteria, it is not used for quantification of an unknown. In addition, any regression range that contains a point that has a saturated fluorescence signal is ignored. A minimum slope requirement can help avoid quantifying samples whose signal falls within a potential plateau region. A user with sufficient permissions may change these values in the Analysis Variables Menu to allow for more or less stringent qualifications.

Step 7: Calculation of Sample Concentration. Sample

signals (background-adjusted median signal) are measured and evaluated against the standard curve for each antibody. Each signal is evaluated against all accepted linear ranges within which the signal falls. For example, in **Figure 3**, a sample with a measured median signal of 25,000 RFU falls appropriately within ranges 2, 3, and 4. In this case, ranges 3 and 4 are accepted as sufficiently linear and sensitive, whereas range 2 does not pass the minimum R^2 value of 0.95 and is not used for calibration purposes.

Within a given antibody set, the concentration for an individual sample is calculated from every range that

passes the stated qualifiers. Up to four concentration values may therefore be calculated for an individual sample within an antibody set. An average of all concentration values is calculated and reported by the VaxArray Analysis Software.
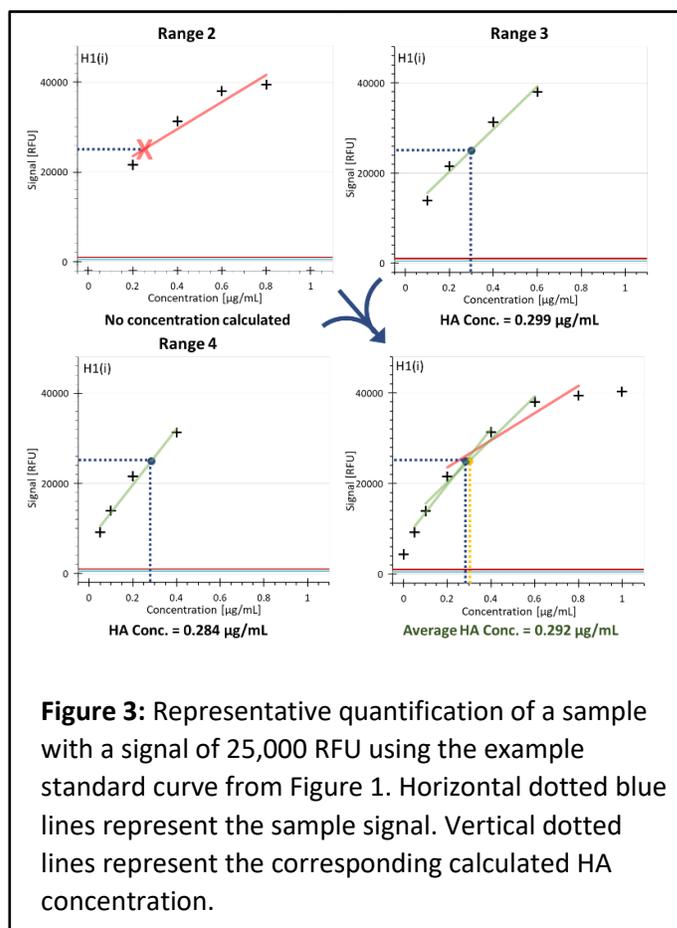

Figure 3: Representative quantification of a sample with a signal of 25,000 RFU using the example standard curve from Figure 1. Horizontal dotted blue lines represent the sample signal. Vertical dotted lines represent the corresponding calculated HA concentration.

ICH Guidelines Compliance. This approach addresses

the ICH guidelines for linearity because it:

i)   Relies on an 8 point calibration curve
ii)   Allows the user to visualize the relationship between signal and analyte concentration
iii)   Effectively limits quantification to linear ranges as defined by linear regression metrics.

Notes. While the default setting in the software uses five (5) 4-point ranges for quantification, the user does have the option to switch to four (4) 5-point ranges. However, this is not recommended as it will generally limit the linear dynamic range of the assay.

In both cases, the user is responsible for validating the assay for its intended use as appropriate.

References:

ICH HARMONISED TRIPARTITE GUIDELINE VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2(R1) Guideline on Validation of Analytical Procedures: Methodology developed to complement the Parent Guideline 2005.