Determining the absolute concentration of high-plex NULISAseq protein targets

TECH NOTE

Introduction

Alamar Bioscience's NULISAseq multiplexed immuno-assays combine industry-leading sensitivity and dynamic range with the ability to multiplex the detection of hundreds of proteins within a single sample. Traditionally, highly multiplexed protein panels relied on relative quantitation methods to measure the relative differences in protein abundance between experimental and control samples. Because standard curves must be generated for each target, absolute quantitation has previously been limited to single analyte assays or low plex panels. This technical note describes a method for calculating absolute protein quantities for a highly multiplexed NULISAseq panel using a pool of target-specific calibrators.

Relative quantification (RQ) with Alamar's normalized protein quantification unit, NULISA Protein Quantification (NPQ), reports differential protein abundances between samples or groups, making it highly valuable for biomarker discovery, disease stratification, and response prediction.[1] Nevertheless, there are situations where reporting protein levels in terms of absolute concentration is useful:

1. **Clinical trials with ongoing enrollment:** Absolute quantification can help ensure data accuracy and continuity when several NULISAseq assay kit lots are utilized, such as over long study periods, large patient cohorts, and/or multiple study sites.

2. **Dose-response:** For dose-response, it is paramount to define specific thresholds. Certain biomarkers may elevate to levels where a therapeutic effect is obtained. Additionally, in drug safety, there is a need to understand when administering a therapy may elevate cytokine levels to undesirable levels.

3. **Biomarker validation:** Absolute protein levels are often necessary to establish different stages of disease or response. Identifying concentration cutoffs without comparing them to a baseline sample is typically required to establish an analyte's utility as a biomarker.

For these applications, Alamar developed absolute quantification (AQ) panel options for some of their highly multiplexed panels.

Absolute quantification using NULISAseq

Conventional immunoassay quantification methods involve constructing a calibrator curve using protein standards, such as an 8-point curve with each standard measured in duplicate or triplicate. This method is viable with single or low-plex assays, such as Alamar's NULISApcr platform, but it is less feasible for hundreds of assays in multiplex, where the cost per sample well is much higher.

To overcome this challenge, Alamar developed a novel approach that enables AQ (i.e., proteins reported as concentrations per sample):

- utilizing a single calibrator sample in triplicate
- a master curve generated for each target as part of the assay manufacturing

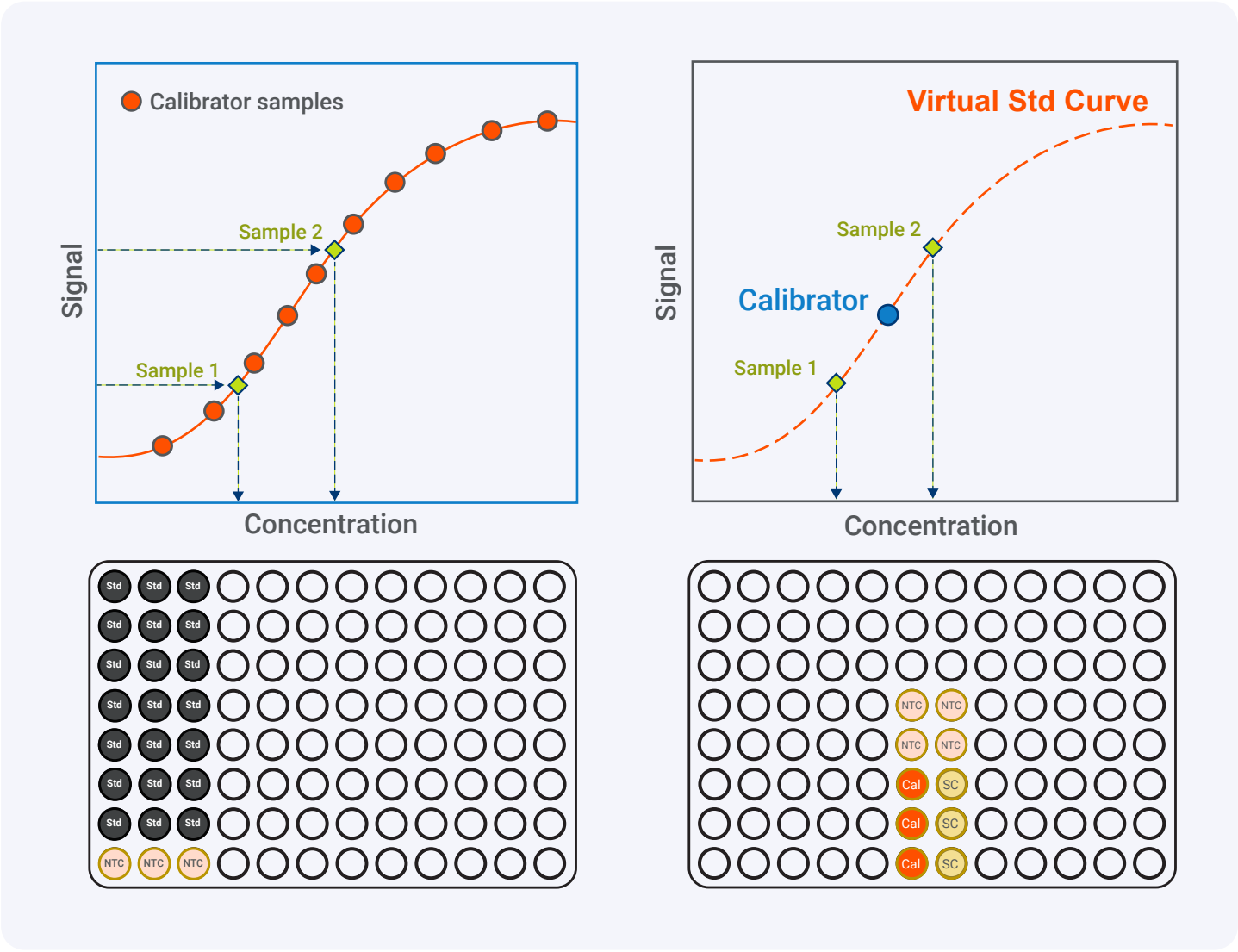

Figure 1. Conventional immunoassay concentration curve (left panels) compared to virtually adjusted curve of NULISAseq AQ panels (right panels). Conventional calibration curves require significant space on the plate, whereas Alamar's approach gives robust absolute quantification while sparing space for samples.

During NULISAseq AQ panel manufacturing, a master standard curve is generated for each target using recombinant antigen and multiple NULISAseq runs (Figure 2.1).

- The antigen pool is diluted two or three-fold at least 15 times to generate a multi-point curve of ≥16 dilution points, excluding the blank.
- A 4-parameter logistic regression is applied for each target to generate a relationship between normalized counts and concentration.

Once the master curve is generated, the calibrator concentration values are established (Figure 2.2).

- The calibrator is composed of a lot-controlled, lyophilized plasma pool.
- The estimated concentration for each target in the calibrator sample is averaged across multiple runs.

The master curve, calibrator signal (in normalized counts), and the calibrator value-assigned concentration are entered into a proprietary algorithm to generate a run-specific virtually adjusted calibration curve for each target (Figure 2.3). The curves are then used to derive AQ values for all samples included in the run.

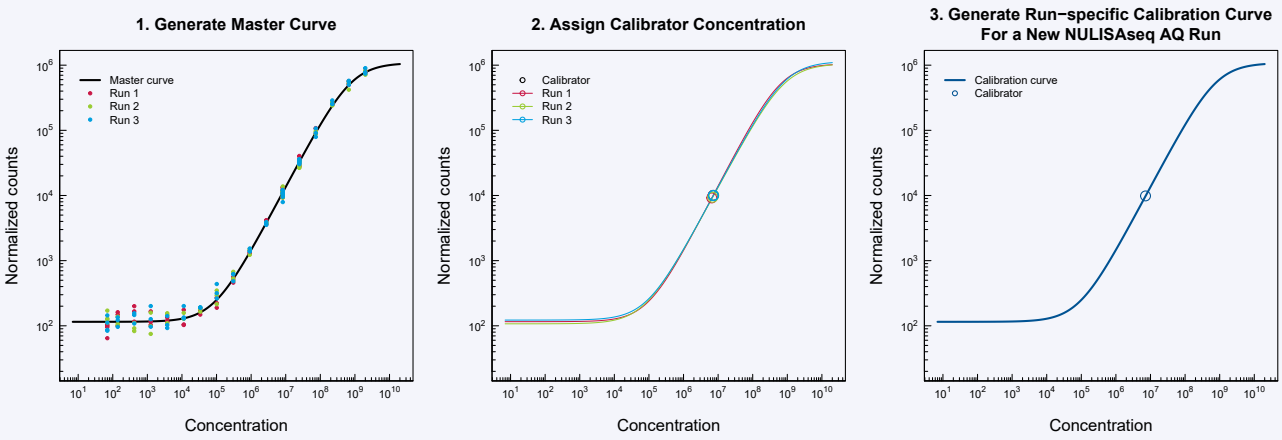

Figure 2. Three-step summary of Alamar's curve generation for NULISAseq Inflammation Panel AQ.

NULISAseq AQ Controls and Quality Control

All NULISAseq panels contain a robust system of controls described in this technical note. Each NULISAseq assay plate includes four negative controls (NCs), three inter-plate controls (IPCs), and three sample controls (SCs). Each sample on the plate is spiked with the same concentration of an internal control (IC) protein.

For AQ versions of the assay, IPCs are replaced with three value-assigned calibrators (CALs), and SCs are replaced with three value-assigned absolute quantification SCs (AQSCs). The CAL samples are used for both inter-plate normalization and target value assignment. The AQSC samples determine assay accuracy and assess intra- and inter-plate coefficients of variation (CVs). The AQSC target values must be within 70-130% of their expected concentration. Table 1 summarizes the quality control (QC) components and associated metrics of the NULISAseq AQ assay.

Table 1. QC metrics for an Absolute Quantification Inflammation AQ run.

QC parameter	Metric	Description
Run Detectability	90%	Percentage of targets that are above limit of detection in at least 50% of samples
AQSC Sample Warning	0	Number of AQSC samples that are permitted to fail QC criteria
CAL Sample Warning	0	Number of CAL samples with sample QC warning
Target Warning	<10%	Percentage of targets with target QC warning
IC CV	<25%	Coefficient of variation of internal control reads across all sample wells
CAL CV	<25%	Coefficient of variation of total reads across all CALs
CAL Target CV	<10%	Median coefficient of variation across all CAL targets
Reads	>1E8	Minimum number of total reads within a plate
AQSC CV	<30%	Coefficient of variation of total reads across all AQSCs
AQSC Target CV	<10%	Median coefficient of variation across all AQSC targets

Individual sample QC is enforced in addition to the overall run QC. Each sample must have more than 500,000 reads, and the internal control for each sample must have at least 1000 reads. Lastly, the internal control of a given sample must be within 40% of the median value generated from all 96 wells' internal controls.

Validation

NULISA multiplex panels are rigorously validated to ensure that each assay performs comparably to quantitative single-plex immunoassays. For absolute quantification, targets must meet strict linearity, dynamic range, and precision cut-offs. Any assay that does not meet these requirements will report a relative NPQ value instead of AQ.

Dilutional Linearity

Every assay in the panel is subjected to an evaluation of dilutional linearity. Thirteen-point two-fold dilutions of plasma samples with spiked-in recombinant antigens are used to evaluate the linearity. Using samples in the dynamic range, at least four two-fold dilutions are measured. Linearity is presented as a percent recovery error:

$$\text{Linearity (\%)} = \frac{\text{[diluted samples]} \times \text{(dilution factor)}}{\text{[first dilution point below ULoQ]}}$$; ULoQ = Upper limit of quantification

A <30% recovery error criteria is used to determine the sample linearity (i.e., AQSC target values must be within 70-130% of their expected concentration).

Dynamic Range, Precision, and Reference Range

We evaluate the dynamic range, precision, and reference range to ensure AQ products report the physiological levels of target assays with acceptable accuracy and precision.

The **dynamic range** of each AQ target is defined as the largest concentration range, for which the intra-plate CV is below 30% and the recovery error is within 30% of the expected value. Multiple NULISAseq runs are carried out to determine the AQ of standards of known concentration using:

- pre-determined AQ master curve parameters
- known CAL concentrations
- CAL normalized counts
- Alamar's AQ algorithm

Precision profile models are fit to these values to represent intra-plate CV as a function of concentration.

Precision is assessed using multiple NULISAseq runs, which include pooled plasma samples spiked with varying concentrations of the recombinant antigen pool. Runs are performed across several lots and instruments. Variance component analysis models are fitted to the data to assess the contribution of various factors to the total CV. All assays in the panel must display intra-plate CVs under 15%, inter-plate CVs under 20%, and inter-lot CVs under 30% within the dynamic range.

For the Inflammation Panel AQ reference range assessment, serum and plasma samples from healthy and disease-state donors are analyzed across multiple plates. The sample set included donors positive for infections, autoimmune disease, neurodegenerative disease, and cancer. Ninety percent of the targets in the AQ panel must be detectable and quantifiable in at least 50% of the samples screened.

Lot-to-lot consistency

Another challenge immunoassays must overcome is ensuring the product performs consistently over its lifetime. Alamar has taken additional steps beyond the stringent QC measures previously described to ensure lot-to-lot consistency:

- Master curves are generated from multiyear antigen pool supplies upon assay manufacturing
- Calibrators and sample controls used in each kit are derived from multi-year supplies
- QC samples are lyophilized for stability and consistency

Lot-to-lot tolerances of sample control accuracies are maintained within 30% CV or less between lots.

The validation data described above can be found in our panel data sheets (RQ data sheet ref., AQ panel data sheet ref.).

Summary

Individual sample QC is enforced in addition to the overall run QC. Each sample must have more than 500,000 reads, and the internal control for each sample must have at least 1000 reads. Lastly, the internal control of a given sample must be within 40% of the median value generated from all 96 wells' internal controls.

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