Filtering, Interpretation and Consideration for Flagged Samples in the SomaScan® Assay

Introduction

The SomaLogic® SomaScan Assay can simultaneously and consistently interrogate thousands of protein analytes for hundreds to tens of thousands of samples for a given study. This is done by leveraging a largely automated process, in addition to powerful data normalization methods, which result in highly reproducible measurements, a large dynamic range, and low median Coefficient of Variation (CV) of approximately 5%.

Performance metrics for the SomaScan Assay have been established to assess the quality of each data set, which may be influenced by several technical and biological factors. The acceptable range for each of these metrics can be found in the SomaScan Quality Statement (SQS), a PDF document created for each study and provided with the SomaScan data file (.adat) during data delivery. One metric is whether the samples are labeled "pass" in terms of meeting expectations for performance metrics, or whether they do not meet expectations and are labeled "flag" instead. The goal of this document is to elaborate on the criterion that classifies a sample as 'pass' or 'flag' based on a mathematical approach, described below.

Key Concepts Related to Scaling and Performance Metrics

Fundamentally, when a sample is categorized as "pass" or "flag", this is largely due to the impact of a series of normalization steps that are applied to the data set. In general, the term 'normalization' refers to the scaling of all data for an experiment to a common place for comparison. Normalization limits the effects of technical variation or systematic experimental biases from a variety of sources. This makes the data from related samples more comparable and downstream analysis more reliable without obscuring the actual biological variance.

Data from the SomaScan Assay utilizes a series of median normalization steps where all analyte measurements for every sample in an experiment are linearly scaled so that, ultimately, they are centered around a common median value. This is accomplished by calculating the median of intensities for each sample, and then scaling the data so that the medians in all samples match.

It is important to note that scaling multiplies the analyte readout (which for the SomaScan Assay is output in Relative Fluorescence Units or RFUs) by a constant value. This linear transformation maintains the variance profile of the samples and does not affect the predictive power for biomarker discovery or model development. In fact, minimal scaling is required when sample collection and processing are consistent, and when there are no technical variations when setting up the assay that might impact the data.

In a laboratory setting, there is always a possibility that unintentional errors or minor deviations in daily activity will result in slight changes to the data. This is easily detected when using pre-defined thresholds that determine the maximum acceptable distance between a measurement and an established median value. In statistics, this median value is often called a reference and used to center the data. For the SomaScan Assay, this is represented as a scale factor, which, simply put, is a ratio of a measurement to the median value of a reference.

When a scale factor for a sample is outside of a predetermined, acceptable range, proceed with some level of caution when using this data point in downstream analyses.

For median signal normalization to work as intended, a fundamental assumption is that the total signal bias (the distance of the center of a sample from the reference) is nuisance variance in the context of a population reference. This can be influenced by whether samples in a study are matrix-matched to the population reference. Alternatively, if the total protein content is inconsistent with the population reference, the resulting scale factors will be shifted to an extreme.

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When samples have not been prepared or stored in ideal conditions (D0004350) or homeostasis of protein levels is disrupted, often to a point of risk of individual survival, the fundamental assumptions for normalization break down. This means that samples from patients with kidney disease, organ trauma, severe immune or inflammation response, people who are pregnant, and pediatric cohorts may result in a higher flag rate.

This document describes conditions which may cause samples to be flagged and offers suggestions for how to best manage them. In the sections below, we outline how a customer may be able to identify the difference between flagging due to technical variation or study-specific flagging. Flagged samples are a useful indicator for pre-screening data to help identify outlier and low-quality samples that may need to be removed from analysis before performing univariate analysis or model development. It is important to review the entire dataset at a high-level to determine whether all data are suitable for including in further analysis before in-depth analysis takes place. Outliers can provide useful information about the sample quality, disease state, or study area; understanding the potential causes of outliers is crucial in deciding whether to exclude them from analysis.

Definition of a Flagged Sample

SomaLogic defines criteria for calling samples as passed or flagged during the normalization steps of the SomaScan data processing procedures. These criteria are detailed in the SOMAscan Quality Statement (SQS) provided with SomaScan data. When all the RFU measurements for a sample are scaled against a reference, or median value, then compared to other samples within a study, the magnitude of scaling of a sample during normalization (when not related to actual biology) can be an important indicator of quality. Ideally most samples in a study should require minimal scaling/adjustment to bring their median intensities in-line with each other. Samples that require extensive scaling/adjustment may warrant further investigation to determine whether including that data in downstream analyses is advisable.

Overview of Data Processing and Normalization

To understand why a sample might be flagged, it's important to understand how the data is generated and standardized, since each step may affect the scale factor of a measurement to the expected reference value. Each control and the steps of the data standardization process are described below and summarized in Table 1:

- **Microarray Feature Replicates:** Prior to data normalization, the microarray slide is imaged, and intensities are extracted for all spots/features on the slide. For each SOMAmer® Reagent, there are replicate features on each array (e.g., 7 total for SomaScan 7K v4.1 Assay). After intensities are extracted, outliers (which could be caused by scratches, for example) are removed and the median intensity for all replicates of a SOMAmer Reagent are recorded in the raw .adat file. Although no actual normalization takes place here, outlier removal and median intensity utilization of replicates makes for more robust data.
- **Hybridization Normalization**: The last step in the SomaScan Assay (before hybridization) involves elution of the SOMAmer Reagents into a buffer containing 12 hybridization control oligonucleotides at concentrations that span the stable readout range on the microarrays (100-100,000 RFU). These controls are used to reduce array variability caused by such processes as sample transfer to slides, hybridization, slide washing, drying, and imaging. By centering the data against a constant median point, this stage of normalization scales the data for each hybridization subarray.
- **Intraplate Median Normalization:** Manages technical variability in Calibrator and Buffer control replicates within a 96-well sample plate. Adjusts for sample well-to-well technical variability among replicates within a plate and dilution bin associated with well-specific occurrences such as bead loss, pipetting, and dilution volume differences. Normalization scales the data for each dilution factor independently so that they are consistent across the plate.

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- **Plate Scale and Calibration:** These two steps minimize differences from plate to plate by first adjusting for overall signal brightness (commonly due to Agilent scanner intensity differences). This is done by applying a single plate scale factor, or a vector of ratios, from the established reference value to the calibrator replicate median on the plate. The calibration step uses the signal of individual SOMAmer Reagents against an established reference value across plates. Plate scaling normalizes the data at a plate level for consistency while calibration involves a SOMAmer Reagent specific signal adjustment across plates to make them more consistent for plate-to-plate variances.
- **Adaptive Normalization by Maximum Likelihood (ANML) to a Population Reference:** Adjusts for technical and inherent sample variability in total signal by centering each sample measurement to the population reference within dilution bin. Controls for technical variation caused by such things as bead loss or pipetting/dilution errors.
- **QC Check:** The final step of data standardization, which verifies results by checking the accuracy of the QC triplicates on the plate against a reference established during assay validation.

TABLE 1 Summary of each step in the data standardization process, purpose, and expectations in terms of scale factors. The 'Location in ADAT' column refers to the row or column in .adat file that includes the calculated metric for the data set.

Standardization Step	Controls for	Expectation	Location in ADAT
Hybridization Normalization	Readout variability (transfer to slides, hybridization, wash, and scan) among samples within a plate	Hybridization scale factor to be between 0.4-2.5	Sample Metadata Column Name: "HybControlNormScale"
Median Normalization (Intraplate)	Technical variability in control (calibrator and buffer) replicates within plate	No defined scale factor parameter	No metrics to label here
Plate Scale and Calibration	Batch effects from plate to plate	Plate Scale Factor between 0.4-2.5 and at least 90% of SOMAmer Reagents have scale factors between 0.6-1.4	ADAT header Row Name: "PlateScale_Scalar_<PlateId>" and "CalPlateTailPercent_<PlateId>"
Adaptive Normalization by Maximum Likelihood	Technical and inherent sample variability in total signal by centering each sample measurement to the population reference within dilution bin	At least 30% of measurements in a sample signal within –2 and +2 standard deviations of reference, where scale factors are within 0.4-2.5	Sample metadata column name: "NormScale_<Dilution>" and "ANML Fraction Used_<Dilution>"
QC Check	Verifies the results by checking the accuracy of the QC triplicates on the plate against the reference established during assay validation	At least 85% of measurements for the QC replicates are within 0.8-1.2	SOMAmer Reagent Metadata: "CalQcRatio_<PlateId>"

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An adjustment that exceeds the specified acceptance requirements causes a sample to be flagged. Flagged samples are classified as having either low overall signal (which require all RFUs to be scaled by a factor above 2.5) or strong signal (scale factor less than 0.4) and indicate how much scaling was applied to the data to bring it in-line with other samples. Sample scale factors can fall outside of the expected range for several reasons such as: deviations from sample isolation or storage protocols, hybridization issues, low or excess protein concentration, deviated sample preparation, or biological factors. A conservative approach is to eliminate flagged samples from analysis, and if one decides to include these samples in the study, one should proceed with caution.

Flag Rates in the SomaScan Assay

The SomaScan Assay is largely an automated, 96-well plate-based assay, followed by microarray hybridization, washing and scanning. SomaLogic performed an internal failure analysis to determine the frequency and cause of flags from failures. In total, 2,000 plasma and serum samples were analyzed, of which 1,900 passed the quality criteria (no flags). Of the remaining 100 samples that were flagged, 95 were due to sample quality prior to the assay itself (e.g., low volume, low protein concentration, or improper storage). Re-running these samples would produce the same result - a flagged sample. The remaining 5 samples were found to be flagged due to a technical anomaly, such as hybridization leakage, loss of material during processing, or inefficient washing. This results in an extremely low technical flag rate of 0.25%. For this reason, SomaLogic does not re-run and re-process a flagged sample; it is unlikely to alleviate the flag and the inefficiency of doing so is detrimental to the assay workflow.

Potential Causes for a Flagged Sample

Assay Notes and Hybridization Scale Factors

When processing samples through the SomaScan Assay, operators can take notes when there are visible deviations from standard process. These notes are transferred to the .adat file. By comparing a flagged sample with any Assay Notes that are included in the .adat file, it may be possible to determine why a sample was flagged.

The Agilent hybridization system for the SomaScan Assay utilizes a glass microarray slide that is designed to hold eight subarrays, one slide for every eight samples. When a sample is dispensed into one of the gaskets, an Agilent microarray slide is then placed on top of the gasket slide containing the samples. If observations such as leaks, bubbles, or lower sample volume are seen in this process, it will be annotated in the 'Assay Notes' column of the .adat file. In most instances, hybridization normalization will adjust for these issues, but extreme over-scaling is risky and might confound the outcome of the data.

Meaning of Assay Notes:

- **Leak/hole, Leak/low histogram**: Refers to low signal in the readout process, particularly when the eluate leaks from the slide gasket the signal from the Agilent readout process, signal is low and tends to enlarge the hole in the center of the readout.
- **Smear:** Refers to some elevated background signal coming from the readout, which are typically well managed by the replicate spots per probe on the printed slides (7 spots for v4.1).
- **Short transfer:** May occur during the first transfer of the sample to the Dilution 1 plate and is a result of there not being enough volume in the tube.

Sample Notes and Quality

If samples are processed by SomaLogic Assay Services, notes regarding the atypical appearance of samples at the time of sample receipt or processing are

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included in the .adat file in the 'Sample Notes' column. Cloudy samples could indicate elevated levels of lipids in the sample, while reddish samples could indicate hemolysis for blood-derived samples.

In some cases of hemolysis or discoloration, we anticipate higher hemoglobin and lower haptoglobin. The hemoglobin to haptoglobin ratio may be used to evaluate sample quality but is not necessary to justify removing samples or for sensitivity analysis. Hemolysis may result in some increased intracellular content as well. When comparing the sample's receipt annotation (red or hemolyzed) with the assay results, neither of these are guaranteed occurrences, but such annotation appears to be linked to increased hemoglobin levels as well as increased overall signal (lower median normalization scale factors). Following the initial normalization steps that mitigate variance from slides and plate-level differences, samples are adjusted to control for subtle differences due to sample quality, but extreme cases will result in normalization scale factors that are outside an acceptable range.

Preanalytical Variation

Proteins can be differentially affected by sample processing and handling methods which can introduce artificial bias and may confound analysis. In the instance of a flagged sample, pre-analytical variation (PAV) may explain why a sample might have been flagged due to collection methods, storage temperatures, processing protocols, or other factors. It is critical to assess any PAV in order to avoid artificial bias in the intended measurements, which could result in misleading data and incorrect conclusions.

SomaLogic's SomaSignal® Tests for Preanalytical Variance (PAV) provide an assessment of sample quality as it relates to time from blood draw to centrifugation (Time-to-Spin), time from centrifugation to decant (Time-to-Decant), and more (SL00000713). Most PAV SomaSignal Tests are ideally suited for troubleshooting flagged samples in EDTA Plasma and Serum; Though not all samples with high PAV will be flagged, from the PAV score, one can get a sense as to whether certain samples are affected by PAV and therefore skewing data. For instance, it can help identify whether samples at certain collection sites or sample groups may have been processed differentially. If high PAV is observed in a data set, it may not be necessary to remove the samples. Each SOMAmer Reagent is affected differently by PAV, and cross-referencing effect size tables at different experimental timepoints or conditions can provide information about which reagents are more affected than others.

Sample Population is Different from Reference Population

Data standardization is often applied to a data set based on a set of assumptions. Deviations from these assumptions can result in misleading or completely erroneous data. For data from the SomaScan Assay, one of these assumptions is that the proteome of samples in the study are biologically similar to the cohort that was used to generate a reference (e.g., adult samples will be normalized against a reference that is also from adults). If these assumptions are not met, a high flag rate may occur, particularly when samples are from a cohort with an exacerbated immune response from extreme sepsis, kidney failure, or pregnancy, for example. There is a wide range of clinical manifestations of immune response that may include a spike in cytokines, widespread tissue damage, or low white blood cell count[1]. When there is an influx of proteins due to an immune response, the total protein content will be different compared to a healthy, normal population that is used for normalization. When total protein is vastly different between the samples in a study and a reference population, scale factors will be at an extreme, and results in a higher flag rate.

Sample Matrix is Different from the Reference Matrix

Another assumption for data standardization is that the matrix of the samples and cohort used to generate a reference are the same. For example, studies with

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serum matrices must be normalized against a reference that was also generated from serum samples. Like severe inflammatory states, total protein content varies for different matrices and anticoagulant types. If total protein content is too different from the samples used to generate the reference values, this will result in a high flag rate as well.

Adaptive Normalization by Maximum Likelihood (ANML)

The section that follows provides a detailed description of ANML and explains why, in certain studies, median normalization against a study-specific reference will be applied instead, particularly where there is a high sample flag rate.

The proteome is dynamic, and normalization should be as applicable to as many clinical conditions as possible without being skewed by significant biological factors. For Adaptive Normalization by Maximum Likelihood (ANML), a sample is compared against a database which contains an external reference population generated from over 1000 human, adult individuals without a known, pre-existing condition. This healthy, normal population establishes what a “normal” range of proteins may be; When an analyte in a sample fall within this range (between –2 and +2 standard deviations of the reference), it is used in the normalization calculation for ANML. The data for the sample is then scaled based on these proteins to center on the median of the reference population. This allows us to adjust the sample on what measurements that are like the population reference, rather than over-adjusting based on proteins that are changing the most in the sample and may be related to our biological factor of interest.

There are several advantages of ANML against an external reference, including that samples are normalized independently from all other samples within a plate or study. Also, outliers in the dataset will not affect the normalization scale factor for other samples. Lastly, it also allows for samples to be added or removed without the need to re-normalize the data.

When samples are flagged after ANML is applied, alternative normalization to a study-specific reference, where a reference is created from the samples themselves, and the samples are centered against that value, may be applied to reduce the flag rate.

Parameters that Define a Flagged Sample

Flagging of a sample occurs when at least one of the following criteria are met:

- Hybridization Normalization Scale Factors less than 0.4, or greater than 2.5.
- Normalization Scale Factors for any dilution group of less than 0.4, or greater than 2.5.
- When Adaptive Normalization by Maximum Likelihood (ANML) is applied, less than 30% of analytes lie between –2 and +2 standard deviations of the reference, suggesting that the samples are too different from the global reference to scale reliably.

RowCheck Column of ADAT Sample Metadata

Each sample that is flagged for any of the reasons described in the section above will be labeled ‘Flag’ in the ‘RowCheck’ column of the .adat files. RowCheck is an overall indicator that the sample meets the normalization scale factor check (PASS) or failed the any scale factor check (FLAG). Samples with scale factors outside of standard acceptance criteria may be filtered from analysis based on this column, especially if scale factors are at an extreme.

ColCheck - Flagging SOMAmer Reagents for QC Controls Only

‘ColCheck’ is a method to track and calculate the number of SOMAmer Reagents that are outside the acceptance range for the QC control replicates on any plate within a study. When the scale factor for any QC replicate on any plate within a study is outside the expected range (0.8-1.2), the SOMAmer Reagent will be labeled as ‘Pass’ or ‘Flag’. This value does not reflect the sample data integrity. These values are based on QC controls only. This approach allows for an evaluation of the assay performance of the QC replicates on a per plate basis but is not used to flag or

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exclude analytes from a study. Instead, it may be helpful to use the Coefficient of Variation (CV) of all QC (Quality Control) replicates across the plates and consider which SOMAmer Reagents may have excess technical noise.

Managing Flagged Samples

Not all flagged samples need to be removed from the SomaScan data set prior to analysis. In general, flagged samples are removed by filtering based on the RowCheck column where the value is equal to 'Flag'. However, it is also reasonable to set alternative thresholds or other methods for inclusion or exclusion of a sample measurement. Samples with scale factors just under or over the threshold may still be scaled appropriately.

Samples that are flagged for a technical anomaly (during hybridization, for example) are typically removed from analysis. Referring to the sample metadata columns, 'Sample Notes' and 'Assay Notes', may provide additional information regarding the failure during the assay run. Depending on the annotation, these samples might be omitted from any downstream analysis.

However, if a sample is flagged because of median normalization, this could be related to biological factors or an issue with sample quality. Extreme median normalization scale factors may not be accurate because RFUs may be over-scaled if the normalization scale factors lie outside the acceptance range. An overly cautious approach would be to completely filter samples that are flagged from a data set. Although, samples that have been flagged for median normalization scale factors may still be informative, particularly if the normalization scale factors are marginally above or below the performance expectation. Combining meta data related to the different sample groups, such as sample collection site or treatment type, for example, could be informative in terms of understanding and interpreting results from the SomaScan Assay.

To evaluate if a sample should be removed from analysis because of skewed normalization scale factors, the outliers, or samples with excess variance, can be evaluated using a variety of methods, such as Principal Components Analysis (PCA), robust outlier analysis (where only measurements beyond 6 median absolute deviations of the median are flagged as statistical outliers, for example), metrics of excess model weight, or other measures. Alternatively, samples with elevated statistical variance may be removed from analysis or subject to sensitivity analysis regardless of acceptance criteria. It may also be helpful to test if the normalization scale factors are correlated with the biological endpoint of interest.

In summary, flagged samples can provide useful information about the sample quality, disease state, or study area. It is reasonable to consider them as outlier samples, which may differ due to biological or technical variables. Understanding the potential causes that classify them as outliers is crucial in deciding whether to exclude them from analysis.

References

1. Fajgenbaum, David C, et all. "Cytokine Storm." *The New England journal of medicine* vol. 383,23 (2020): 2255-2273. doi:10.1056/NEJMra2026131

Additional Resources

- SomaScan Assay V4.1 (SL00000572)
- SomaScan v4.0 and v4.1 Data Standardization (SL00000442)
- Calibration and Normalization Technical Note for Single Dilution Kits (D0005157)
- Pre-analytical Variation (PAV) Tech Note (SL00000713)
- The SomaScan Assay: Recommended Sample Handling and Processing for Core Sample Types (D0004350)

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Flagged Sample in the SomaScan Assay Tech Note

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