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Bioanalysis

New approaches for biomarker stability determination in regulated bioanalysis: trending, bridging and incurred samples

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Aim: Determining the stability of biomarkers continues to present challenges. Disease states, complex matrices and differences between recombinant and endogenous analytes require new approaches to maintain stability and measure it. In this report, we determine stability for two assays using trending and statistical analysis. **Methodology & results:** Monitoring trends helps identify out of specification measurements and determine whether concerns are due to the stability of the analyte. We also describe challenges presented when measuring arginase activity in human sputum, a complex matrix, for respiratory diseases. We controlled preanalytical protease activity and collection heterogeneity and monitored incurred sample stability to improve stability of arginine. **Conclusion:** These new approaches to achieving and determining biomarker stability may provide solutions for increasingly complex biomarker measurements.

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Stability is the measure of intactness of an analyte (lack of degradation) in a given matrix under specific storage and use conditions relative to the starting material. The goal of testing the stability of biomarkers is to determine the stability of the protein in the intervened environment of the study matrix and disease being studied. Although US FDA bioanalytical method validation guidance addresses the use of biomarkers, the stability method is focused on pharmacokinetic (PK) assays. Currently, due to lack of specific biomarker guidance documents, stability evaluation is often performed in one of two ways: using individual or pools of patient-derived samples or validation samples spiked with a purified/recombinant analyte [1].

Using a purified or recombinant protein to determine stability, regardless of the matrix used, is problematic because the purified protein likely does not mimic the endogenous analyte. Differences in folding and post-translational modifications can heavily impact the stability of the protein differently from the endogenous protein. In a comparison between purified spiked IL-13 and patient samples, poor recoveries were shown for spiked recombinant IL-13 at month 5, whereas patient samples showed stability up to month 15 [2].

The use of patient pools or individual samples is also problematic because the stability of these samples is determined by intermittent analysis in batches in conjunction with freshly prepared controls. Each batch is judged separately, and systematic errors such as a change in reference material or critical reagents may affect a single batch but not be indicative of changes in the analyte's concentration [3].

There is also evidence that disease states affect stability of proteins due to differential regulation of proteases and protein folding. In the case study of TGF-B, samples spiked into normal urine showed higher recovery after one freeze thaw cycle compared with samples spiked into diabetic urine [2].

At last, biomarker analysis is also becoming more complex with specialized matrices and nonprotein biomarkers such as micro-RNA. A true purified standard of miRNA is often unavailable, and disease states can alter the stability of miRNAs further complicating stability determinations of these biomarkers [4,5]. Complex matrices such as sputum can also complicate stability assessment due to proteases and heterogeneity of patient samples which may not match a recombinant spiked sample.

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In this study, we present three case studies using nontraditional approaches to determine or maximize the stability of biomarkers: trending analysis, computational and statistical analysis and assessing preanalytical sample preparation and using incurred samples for a complex matrix. While these approaches are not yet common in a regulated bioanalytical environment, they may address the challenges highlighted above for increasingly complex biomarker measurement.

Methods

Case study 1: use of trending to evaluate the stability of endogenous quality control

We measured a protein biomarker found endogenously in serum (TGF-B1). The intended purpose of the assay was the exploratory evaluation of the biomarker in clinical samples. Due to the exploratory nature of the data, it was decided that an ELISA kit (Somru BioScience, PE, Canada) will be utilized along with the inclusion of one endogenous quality control (QC) (human serum pool from a commercial source). An additional QC was prepared by spiking endogenous QC with recombinant protein (20 pg/ml). Both QCs were designated for stability testing at −80°C and were measured over 20 stability runs (monthly) during validation, and the mean value was used as nominal concentration. The calculated mean and standard deviation (sigma) from the 20 runs were used to set the following criteria for monitoring trending of the stability runs:

- A single point outside of 3 sigma of mean;
- Two points outside of 2 sigma;
- Four points outside 1 sigma;
- Four points in a row on the same side of mean centerline.

Case study 2: utilizing statistical approaches to differentiate true stability failure versus lot-to-lot kit variability

The assay was an internally developed sandwich ELISA (commercially available, Somru BioScience) using purified recombinant IL-13Ra (Cho cells) as a reference standard that utilizes mouse monoclonal antibody as capture and goat polyclonal antibody as detection. The assay range was 50–3000 pg/ml. This biomarker was utilized as the pharmacodynamic biomarker to support clinical study.

The assay utilized five lots of kits (Lots #1–5) and three lots of calibrators. Calibrator lot A was used for kit lot number 1, calibrator lot B was used for kit lot numbers 2 and 3, and calibrator lot C was used for kit lots 4 and 5. An endogenous human serum sample (commercial source) and spiked endogenous sample was stored at −80°C as the stability samples measured in validation and over a 3.5-year study.

To accurately analyze stability data generated using multiple kit lots, we used the following computational 'bridging' approach originally developed by Feng *et al.* [6]. In brief: we generate a 'reference' calibration curve (RCC) using validation data (20 runs); generate the measure of distance for each calibration curve from the RCC, assuming that lot-to-lot differences are due to variability/inaccuracy in the standard analyte; using this process, we calculated a correction factor (S, shift factor) for each batch to normalize the entire calibration curve and recalculate the stability results. The S factor is determined through nonlinear regression with the other 4-PL regression parameters.

Case study 3: sample processing stability for the measurement of arginase activity

An enzyme activity method was developed for arginase in human sputum samples. The method involves incubation of L-arginine solution (50 ul) to heat-activated sputum lysates (50 ul). A minimum dilution of tenfold is required. The amount of urea generated from this reaction is then determined by a spectrophotometric assay based on a reaction with α-isonitrosopropiophenon. The arginase activity is calculated from a standard curve for urea (1–30 µg urea). The results are expressed in absolute terms as units/mg protein. One unit is defined as the enzyme activity that catalyzes the formation of 1 µmol urea/min. If desired, the specificity of the assay can be confirmed by showing that the specific arginase inhibitor inhibits the signal.

To improve stability of enzyme activity and homogeneity of the complex sputum matrix, we tested dithiothreitol (DTT) at 5 mM, Triton X100, protease inhibitor cocktail (Somru BioScience) and mechanical homogenization (vortexing). Due to the presence of *in vivo* components in the study sputum samples, which would affect stability, we used incurred samples instead of spiked QCs. Thus, sputum stability samples were produced via expectoration (commercial source) without additional recombinant spiking. Due to the time to receive incurred sputum samples

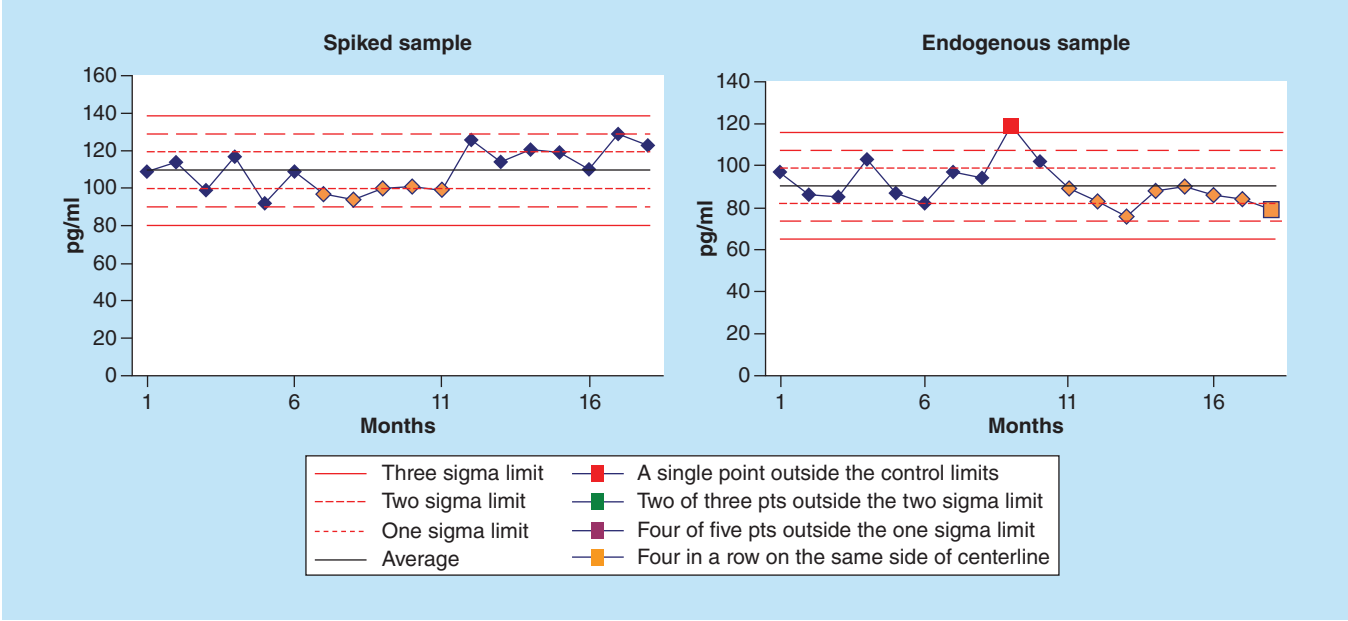

Figure 1. Use of trending analysis to investigate stability. Measurement of TGF-B over 20 measurements in a recombinant spiked stability sample and endogenous stability sample. The endogenous sample differed from the spiked sample and while measurements met batch acceptance criteria, a trend for decreased stability was seen in the last eight measurements.

and perform processing, stability samples were frozen at −80°C for 24 h after collection and used as the zero time point (T0). The results of these processing steps on stability are discussed later.

Results

TGF-B1 stability during validation with trending analysis

A total of 20 measurements of the stability samples in validation were taken and plotted against the mean and standard deviation of the results. The mean and standard deviation are usually recalculated after a set number of runs (e.g., every 20 or 40 runs), but the decision rules remain the same. Due to the exploratory nature of the work, the assay acceptance criteria was set at 30% of nominal concentration for both system suitability and the stability assessment. If batch acceptance criteria were met, the data point was added to the control chart for trending analysis. Triggering of any of the four decision rules in the control chart warrants further investigation (root cause, corrective action). QCs from spiked protein differed significantly from the endogenous QCs. Our control chart rules (four in a row on the same side of mean centerline) were flagged in the last seven data points of the endogenous QC (Figure 1). The endogenous QC recovery was consistently below the mean line indicating endogenous QCs may be unstable. The trending alerted the scientist to the need to perform stability testing in more close intervals and accelerate the sample analysis to avoid potential stability issues. This decrease in stability would likely not be identified without trending analysis as each individual measurement met acceptance criteria.

Computational analysis of IL-13Ra stability

We measured IL-13Ra in a long-term study (3.5 years). Stability samples failed batch acceptance criteria at 36 months and afterward and were all below the baseline measurement at T = 0, initially suggesting deteriorated analyte stability (Table 1). We observed the stability data against time and determined failed stability correlated with change in reference standard lots. Further analysis showed the calibrator lot changes led to a shift in the calibration curves which would alter stability measurements. This suggested the out of criteria stability measurements were not due to a decline in analyte stability.

It was discovered that the kit lots 4 and 5 (which both used the new lot of calibrator C) significantly underestimate the analyte concentration. Both the kit lots utilized the same lot of calibrator standard. In addition, the long-term stability fails to meet the acceptance criteria (± 20% bias) when using kit lot 5. Using the computational approach highlighted above, we determined a correction factor based on the RCC. We calculated the difference between

Table 1. Stability samples of IL-13Ra over five kit lots during the 3.5-year study.

	Time	t=0	3 d	7 d	1 m	2 m	6 m	9 m	12 m	18 m	24 m	36 m	39 m	40 m	41 m	42 m
Endogenous sample	Conc pg/ml	114	104	97	119	122	101	98	109	129	101	83	89	82	80	85
	% bias		−9	−15	4	7	−11	−14	−4	13	−11	−27	−22	−28	−30	−25
Endogenous sample w/ correction S	Conc pg/ml	114	104	97	119	123	101	98	109	129	101	94	101	94	92	97
	% bias		−9	−15	4	8	−11	−14	−4	13	−11	−18	−12	−18	−19	−15

Table 2. Stability samples with dithiothreitol and protease inhibitor before storage.

Preanalytical condition	Sample ID	Concentration (mU/mg)		
		24 h (T0)	72 h	7 days
− DTT + protease inhibitor	1	397	388	234
− DTT + protease inhibitor	2	354	328	276
− DTT + protease inhibitor	3	376	209	188
+ DTT + protease inhibitor	1DP	510	488	519
+ DTT + protease inhibitor	2DP	432	409	455
+ DTT + protease inhibitor	3DP	396	401	421
+ DTT − protease inhibitor	1D	488	470	432
+ DTT − protease inhibitor	2D	398	311	235
+ DTT − protease inhibitor	3D	401	302	198

DTT: Dithiothreitol.

Table 3. Long-term stability maintained to 2 months with dithiothreitol and protease inhibitor.

Sample	24 h	72 h	7 days	1 month	2 months	% bias 2 months
1DP	510	488	519	444	472	7.45
2DP	432	409	455	371	384	11.1
3DP	396	401	421	328	331	16.4

the RCC and each of the curves for stability measurements. Importantly, the calculated correction factors S were applied to each calibration curve to shift the entire calibration curve to whichever concentrations were determined. This nonlinear shift is a more non-biased bridging approach to determining biomarker stability for long-term studies.

Bridging of kit lots using a correction factor determined by robust computational analysis demonstrated biomarker sample stability was maintained to 42 months. Failed stability samples before correction are shown in red.

Arginase activity in sputum

Endogenous arginase activity in incurred stability samples of sputum demonstrated significant deterioration of arginase activity after only 7 days of storage at −80°C. (Table 2). This was likely due to presence of proteases and ureases present in saliva which mixes with sputum. In addition, the three stability samples showed significant variation likely due to the viscous and heterogeneity of the sputum matrix.

To address these concerns, we performed preanalytical processing of stability samples prior to storage and time 0 measurements. We added protease inhibitor to inhibit urease activity and 5 mM DTT to address sample heterogeneity. This improves the reproducibility of replicate measurement as well as reproducibility of measurement of the stability sample over time. The active component in DTT is a sulphydryl group, which cleaves disulfide bonds in the mucus and opens enzyme active sites for arginase [7]. A higher amount of DTT may impact the components (proteins, antigens, lipids, metabolites, nucleic acids) of the sputum and impact the assay. Samples were also diluted tenfold in buffer to reduce viscosity and with these additions, stability of samples was extended to 2 months at −80°C (Table 3). 3 months stability data, however, were inconclusive: two samples failed by about 22%; one passed within 10%.

Discussion

The measurement of biomarker stability using the batch-specific calibration curve model suffers from significant limitations as in most cases there is a limited number (5–7 nonzero points) of calibrators per plate. Often the calibrators are made by using serial dilution which may exacerbate any error. QC samples are not often able to detect variability in calibration curve model as the criteria for QC acceptance range can be 40% (± 20%) to 60% (± 30%). In this report, we present two case studies where trending analysis was used to investigate reasons for failed stability measurements. In these instances, trending analysis demonstrated benefits over the current standard of using batch acceptance criteria.

New FDA bioanalytical method validation guidance encourages use of QC trending analysis for monitoring assay performance [8], and the Critical Path Institute has recently recommended using trending and control chart analyses for monitoring stability of biomarkers [9]. Control charts graph assay results longitudinally over repeated measurements and flag values statistically in relation to recent results instead of separately [10]. This triggers closer monitoring of the assay and allows investigation of root causes to determine whether out of control behavior is in fact due to sample stability.

This practice is well established in clinical chemistry where Westgard rules are used to establish warning levels (e.g., a single result outside three standard deviations of the mean) [11]. However, strict adherence to Westgard rules could cause rejection of data acceptable by bioanalytical standards. In our case study, we made four decision rules modified from Westgard rules. Similar to the rules set by Bruijnsvoort *et al.* [10], the decision rules allow for occasional failure due to random and systematic error allowable by current regulatory guidelines and prompting an investigation if triggered instead of sample rejection. The rules can also be fit-for-purpose by the lab to accommodate the use of the biomarker, number of runs and variability of the assay.

For endogenous samples or incurred samples, it is frequently challenging to determine T0 of the sample for stability analysis as the actual concentration is not known and may not be accurate as delay in sample receipt before storage may affect stability. Trending analysis can also help in this regard as the trend of degradation can be used in place of comparison to T0.

A limitation of using trending analysis for stability is that formal stability analysis is traditionally run at specific time intervals (e.g., monthly), which limits the number of data points analyzed. One solution is to monitor the endogenous and spiked QCs run with each batch. While not stability testing *per se*, this analysis can provide a more substantial data set to show a trend in degradation and helps compare stability of recombinant analyte versus endogenous analyte.

In our case study using QCs, we saw a trend for decreased stability, but this trend was not seen in recombinant sample. This highlights how the stability of samples with a spiked analyte in matrix can differ from the endogenous analyte. Stability of biomarker assays thus should be assessed with both recombinant and endogenous samples. Also, stability should be monitored with trending analysis before acceptance criteria for stability fail as a degradation of the biomarker can be detected earlier. Trending coupled with monitoring of batch QCs is likely to give the most accurate assessment of stability for biomarkers.

The benefits of using trending analysis were further demonstrated in a recent case report by Bruijnsvoort *et al.* where the authors measured hepcidin in serum with an endogenous QC on each assay run giving a robust dataset of 144 data points over 3 years [10]. A downward trend at 2 years triggered decision rules, which seemed to correlate with a new reference material lot. However, cross-comparison of stocks ruled this out, and the downward drift continued to 3 years. The trending methodology using batch QCs, thus helped them set long-term stability of 2 years.

Computational bridging tools to asses stability in long term studies

The objective of this investigation was to identify the root cause of stability failure and suspected low concentration of IL-13Ra in stability samples analyzed after 36 months.

In general, immunoassay data are known for high variability from different sources such as reagent quality, operator difference, sample composition and reagent/analyte stability. It is almost universal practice to utilize a standard curve in each ELISA plate and generate a calibration function. In this fixed-effect method, the concentrations of samples are then calculated using this calibration function [3,12]. The results of the unknown samples are highly sensitive to the performance of the calibration model in each plate. If the calibration curve fails, no results can be generated. Biomarkers studies tend to run for a longer period and very often it is necessary to compare studies

that were conducted over a span of years. In many cases, establishing sample stability can be very difficult, if not impossible. if there is high variability in the calibration curve.

In order to address these challenges, some have proposed 'mixed-effect' or 1-step analysis methods where statistical methods are used to incorporate previous regression curves into concentration determination [13]. Similarly, while facing the problem of kit-to-kit variability/calibrator lot-to-lot variability, some labs have responded by using correction factors to bridge different lots together across a long study [14]. The use of correction factors without appropriate statistical methodology can result in erroneous results as it assumes that variability across batches are constant and the variability across the concentration ranges is homogenous. Here we present bridging via the mixed-effect statistical approach that considers the kit lot-to-lot variability and variability from each batch before applying the correction factor.

In this case study, the correction factors ameliorated the effects of changes in reference material and demonstrated was biomarker was not instable. This method of determining stability is not yet widely used, especially in regulated bioanalysis. However, biomarker working groups have recently recommended statistical approaches for biomarker assays such as for determining total allowable error [9]. The S factor method and subsequent software initially developed by Feng *et al.* [6] could potentially be adapted for regulated bioanalysis to bridge assay validation, sample analysis and accommodate audit trails.

Due to the fit-for-purpose nature of biomarker assays, this approach is perhaps best suited for exploratory or secondary end point biomarkers. For primary end point assays, better control of the calibrator or biomarker kit lots could prevent shifts in calibration affecting stability runs and avoid the need for data adjustment.

One limitation of this approach, due to the variability of biomarker calibrator material, is that it is difficult to know if the assay runs used to form the reference curve are based on problematic calibrator. Performing parallelism during method validation to verify the calibrator accurately represents endogenous analyte may ameliorate this concern.

Arginine/arginase stability in complex sputum matrix

Sputum is an example of a complex matrix. It is produced by a patient by mouth from mucus from the airways and thus is combined with saliva [15]. Sputum collection usually cannot be standardized as different amounts of saline and coughing are needed for each patient to produce sputum. Due to these reasons, sputum is very heterogeneous (mucoid, purulent, mucopurulent, blood-streaked, watery, viscous) and contains proteases found in saliva.

Nevertheless, sputum is becoming a matrix of choice for respiratory diseases. Sputum biomarkers can be indicative of current disease state, predictive of future outcomes and might identify mechanisms and provide treatment targets, trial end points and objective clinical monitoring tools [16]. Arginase activity in sputum specifically is an essential biomarker for cystic fibrosis as increased arginase activity has been shown in these patients to decrease L-arginine levels and subsequently decrease nitric oxide levels in the airways [17].

To accurately measure stability in sputum, we elected to analyze incurred samples instead of spiked QC samples. High and low concentration stability samples from QCs based on the standard curve would not reflect the heterogeneous nature of collected sputum and would not be impacted by preanalytical processing in the same way as study samples. Incurred sample stability which are incurred samples measured over an extended time would accurately reflect these variables and can be chosen at high and low concentrations similar to how study samples are chosen for parallelism assessment of biomarkers. Incurred sample stability is recommended in cases where enzymatic degradation of an analyte is expected as is the case in sputum with proteases [18].

Preanalytical solutions were used to achieve stability in sputum beyond 7 days. To achieve specimen to a homogenous state, in other words, evenly distributed suspension at the clinical site for dispensing into multiple aliquots, two approaches were utilized – mechanical homogenization (vortexing) or chemical (DTT). Even with our preanalytical processing, stability after 3 months was inconclusive and variable – demonstrating the value of using incurred samples to truly asses biomarker stability. Further stability considerations like pH, buffer composition and temperature activation of enzyme activity before storage have been shown to alter arginase activity [19].

Future perspective

While these methods are not widely used for biomarker immunoassays, particularly in regulated bioanalysis, our case studies demonstrate they have utility for determining biomarker stability or prolonging stability. As biomarkers are used more often to determine therapeutic effects, more extended studies will necessitate the use of novel

approaches. In this regard, groups such as the Biomarker Assay Collaborative Evidentiary Considerations Writing Group, Critical Path Institute (C-Path) suggest the use of trending analysis for determining biomarker stability.

Executive summary

- The use of trending analysis and computational tools to bridge reproducibility are useful investigational tools when determining biomarker stability. Trending, especially if assessed with quality controls on each batch, helps determine whether failed stability is due to stability of the analyte or other assay parameters (lot or critical reagent changes, disease conditions, etc.).
- Computational tools, which bridge lots or standard curves, can address reproducibility affecting biomarker stability in a nonbiased manner if it is determined that failed biomarker stability is not due to analyte stability itself.
- For complex matrices such as sputum, stability can be increased by careful consideration of preanalytical sample preparation such as use of protease inhibitors, temperature control and sample dilution. Use of incurred study samples to supplement recombinant stability samples may better represent the degradation of the endogenous protein.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical conduct of research

All humans samples (serum and sputum) were purchased from a commercial source. Institutional review board approval and informed written consent were obtained by the vendor prior to collection and shipment of the samples.

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• **Provides an in-depth demonstration of the use of trending and control charts which can be applied to biomarker stability.**

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