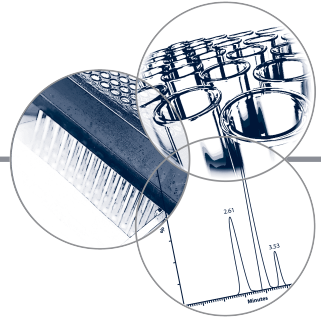

Case studies from the use of commercial biomarker/protein test kits

Large-molecule drugs (therapeutic proteins, peptides, various forms of antibodies) are more frequently being seen in drug-development pipelines, the majority of which are measured using immunochemical/ligand-binding techniques. The assays utilized for analysis of large-molecule drugs rely heavily upon the quality of the components (e.g., reference materials, antibodies) that are critical to the performance of the assays. Commercially available research-grade materials and kits offer a convenient and simple solution, but also present some unique challenges. This article will explore some examples of issues encountered while employing commercially available kits and reagents.

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Large-molecule drugs (therapeutic proteins, peptides, various forms of antibodies) are more frequently being seen in drug-development pipelines. These types of analytes pose unique challenges when it comes to bioanalytical method development. The majority are measured using immunochemical/ligand-binding techniques that are based on indirect measurement of the analyte using capture reagents (e.g., antibodies). These capture reagents are typically developed specifically against the analyte of interest, but can vary in the characteristics (e.g., specificity) and quality. Assays for many **biomarkers** also comprise a large component of drug-development programs and these markers are frequently analyzed using similar techniques and reagents [1–4].

Commercially available **research-grade materials and kits** offer a convenient and simple solution as antibody pairs and key reagents (e.g., conjugates) have already been developed by the manufacturer. The challenges of research-grade kits have been well described elsewhere [5,6] but typically they are designed for research and development applications, which make them very flexible for work in a research and development setting and discovery work as they are usually the first to the market and may be used with multiple matrices. However, they are not intended for drug-development programs. In the optimal situation, the kit manufacturers report the results from an in-house/abbreviated validation at the time of manufacture but frequently the validation expectations are not stated and this documentation is incomplete or absent in the kit inserts. In most cases research-grade kits are designed for measurements in 'best matrix' situations or buffers, not for normal or patient matrices – the ability of the methodology to accurately recover the target analyte in the target matrix may only have been tested in a minority of lots.

This article will provide some further examples of issues encountered while employing commercially available kits and reagents.

Case study 1: reference ranges & matrix effects

In many cases, the **reference material** provided in a commercial kit is not suitable for use during the validation of a method for the quantitative determination of a biomarker and an alternative source for the material must be found. The substitution of the alternative reference material for the reference material supplied with the kit often requires additional testing to ensure its compatibility with the assay. One such instance we have encountered is the bioanlytical method for the determination of parathyroid hormone (PTH) 1–84 in human plasma. PTH 1–84 is an endogenous polypeptide endocrine regulator of calcium and phosphorous concentrations in the body.

Commercial immunoradiometric assay (IRMA) kits were obtained from Scantibodies and the product literature indicated that the supplied calibrators are prepared in stabilized human serum containing sodium azide. The literature further indicates the lyophilized material supplied in the kit should be reconstituted with distilled or deionized water prior to use. A recombinant PTH 1–84 reference standard was purchased from National Institute for Biological Standards and Control (NIBSC) due to the inadequate certificate of analysis for the kit standard. Given the endogenous nature of

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Key Terms

Biomarker: A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention.

Research-grade kit: Prepared kits designed for the measurement of an analyte, designed primarily for research and development purposes that have limited validation at the supplier.

Reference material: Material characterized for one or more specific properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty and a statement of traceability.

Incurred sample reanalysis: The widely used and accepted procedure by which the quality of all types of bioanalytical assays are evaluated through examining the repeatability of concentration values for a subset of samples from individual preclinical and clinical studies.

the peptide, calibrators could not be prepared in serum as they are in the IRMA kit. Therefore, a suitable surrogate matrix had to be identified for use with this method.

Initial testing compared the kit calibrators prepared as indicated in the literature with kit calibrators prepared in three different surrogate matrices. The surrogate matrices tested were 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), 2.0% BSA in PBS, and SeraSub® (CST Technologies), a synthetic polymer in buffered solution which is physically equivalent to serum and plasma with respect to specific gravity, viscosity and osmolality. Following this comparison, 2.0% BSA in PBS was selected as the surrogate matrix for the preparation of calibrators using the NIBSC reference material.

During the qualification of the method, a bias affecting low-concentration samples was observed. Further examination indicated parallelism was not achieved using 2.0% BSA in PBS and therefore, another surrogate matrix needed to be identified. To find a matrix more similar to the actual samples in plasma, we sought to remove the endogenous PTH 1–84 from human plasma and use that depleted plasma to prepare calibrators. Initial testing demonstrated the method for depleting plasma of its endogenous PTH 1–84 was successful (Figure 1). Calibrators were then prepared in both 2.0% BSA in PBS and the depleted plasma and a comparison of the two sets of calibrators demonstrated the differences between the two matrices, particularly at the lower end of the curve (Figure 2). Furthermore, when the concentrations of plasma quality control (QC) samples calculated using each set of calibrators were compared, the high bias seen in the buffer calibrators resulted in a low bias in the QC sample concentrations (Figure 3). The calibrator matrix was changed to treated, depleted human plasma and the method was validated for the quantitative determination of PTH 1–84 in human plasma. The use of treated human plasma allowed for a matrix quite comparable to the actual sample and QC matrix and reduced or eliminated the bias seen with other, more dissimilar surrogate matrices.

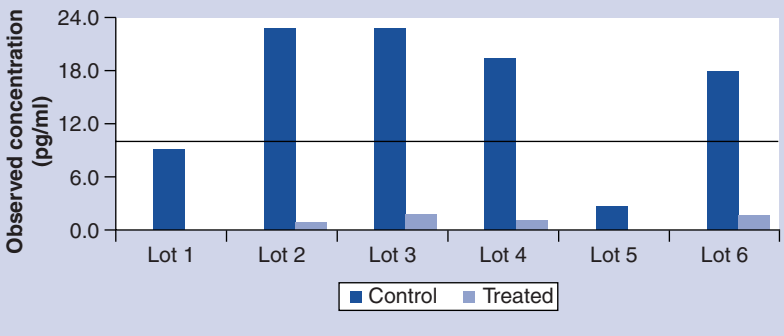

Figure 1. Endogenous parathyroid hormone 1–84 concentrations in control and treated human plasma. LLOQ of assay is indicated by a solid line.

Case study 2: bridging reference material lots

Phase II and III clinical studies can span several years, particularly for trials in oncology and large-molecule drugs whose half lives can be prolonged relative to their small-molecule counterparts. Samples from these types of trials can arrive at infrequent and irregular intervals due to the nature of patient recruitment and shipment of samples. For vendors, the unpredictability produces logistical challenges when purchasing and stocking commercial kits for analysis. Vendors of bioanalytical services are reluctant to stock large numbers of kits or kit components when the established stability period is unknown or shorter than the interval between sample shipments. It is not prudent to purchase a stockpile of kits and the specific lot number of a kit or component will change over time from the lot originally used for the validation of the method. It is critical to think ahead and establish processes that will monitor the performance of the assay after prolonged intervals where the assay is not being performed and substitutions of kit materials.

This is particularly critical when managing changes in the lots of reference material supplied with kits. An illustration of this comes from an ELISA assay that was developed using a commercial research kit for hepatocyte growth factor, a paracrine cellular growth, motility and morphogenic factor in human serum. The assay was validated to support a set of Phase II/III studies that was run for 3 to 4 years. The initial validation was performed over a short period of time. In line with the standard expectations for ligand-binding assays the performance of validation QCs met the conventional acceptance criteria of ±20% relative error, ≤20% coefficient of variation (CV).

Over the course of the study kits were received with a set of calibrators that were prepared by the manufacturer and required no further reconstitution or dilution. A set of calibrators was prepared with each new kit preparation. During the course of the studies more than one lot of kit was purchased due to the length of time the

studies were run. A process was established to ensure consistency between calibration curves whereby the performance of calibration curve and QC samples from the older kits were compared alongside newly prepared calibration standards on the ELISA plates that accompanied the new kit.

FIGURE 4A shows the results of a successful comparison of standard curves from two separate kits. The standard curve and QCs prepared with the reference material from the two kits were nearly overlapping in their performance and matched the performance obtained in validation. Likewise, the performance of QCs prepared from the same reference material performed well, within the performance expected from the validation. Consequently, it was possible to move forward with sample analysis with the new lot of kit and reference material, confident that the back-calculated concentration of a sample read off the initial curve would yield similar results in assays conducted with the two standard curves. FIGURE 4B, on the other hand, displays the standard curve from three different kits during a qualification assay conducted at a later date. It is evident that the curve prepared from the intermediate kit is not performing in the same manner as the curves from the other two kits, particularly in the middle of the curve range. This resulted in QCs prepared with the intermediate kit reference material underrecovering (>20%) compared with the initial standard curve. Had the study progressed with analyzing samples against the curve prepared with the reference material from the intermediate kit, the concentration values generated would potentially have been found to be >20% lower than if they had been measured against the first kit's standard curve. The importance of developing a standard procedure for the qualification of different lots of kits in this case is clear. A clear recommendation from this illustration is that a clear qualification process should be established *a priori*, preferably during method development, for ensuring consistency in the performance of reference materials and for qualifying new batches of reference material as they become available for use.

The benefits of these processes may equally be derived for critical reagents used in ligand-binding/immunoassay techniques. Indeed, while reference material used in a study will remain unchanged, secondary reagents (e.g., antibodies, antibody conjugates) more frequently change depending upon their ease of production, speed of use, storage stability and fluctuations in availability for commercially acquired reagents. Two recommendations are to communicate frequently with your suppliers to benefit from advance information on availability of key reagents, changes in processes, and to understand their QC processes. For primary vendors it is prudent to consider having the quality assurance department visit the supplier and audit their processes and quality systems to ensure that a reliable and consistent supply of materials can be obtained for the duration of a clinical study.

The variability between different curves and kits also underscores the value of including **incurred sample reanalysis** as part of any long-term projects and may be considered of use in some biomarker studies. An alternative to incurred sample reanalysis for an analyte/biomarker that has constitutive levels is to plan to include a QC sample that is a matrix lot or pooled matrix lots with an endogenous value [7]. This lot or pool may be run in all assays to provide further confirmation that the assay is performing in a similar manner across time and that there

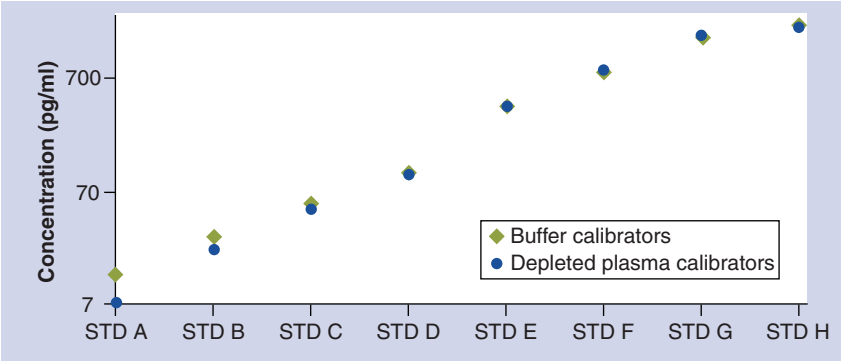

Figure 2. Comparison of calibrators prepared in buffer or in depleted plasma.

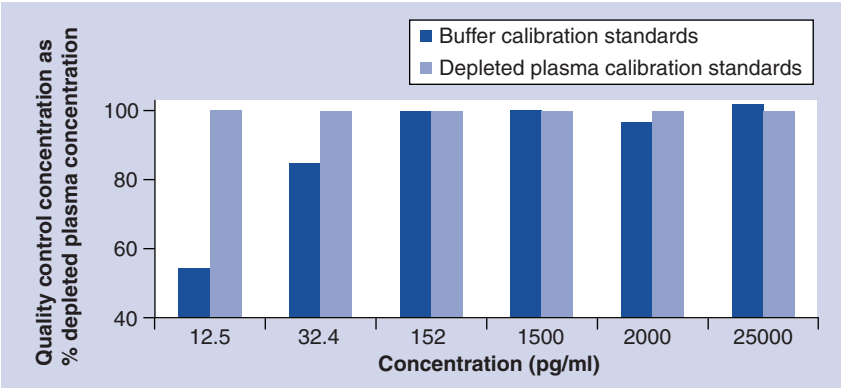

Figure 3. Quantitation of quality control samples using standards in buffer or depleted plasma.

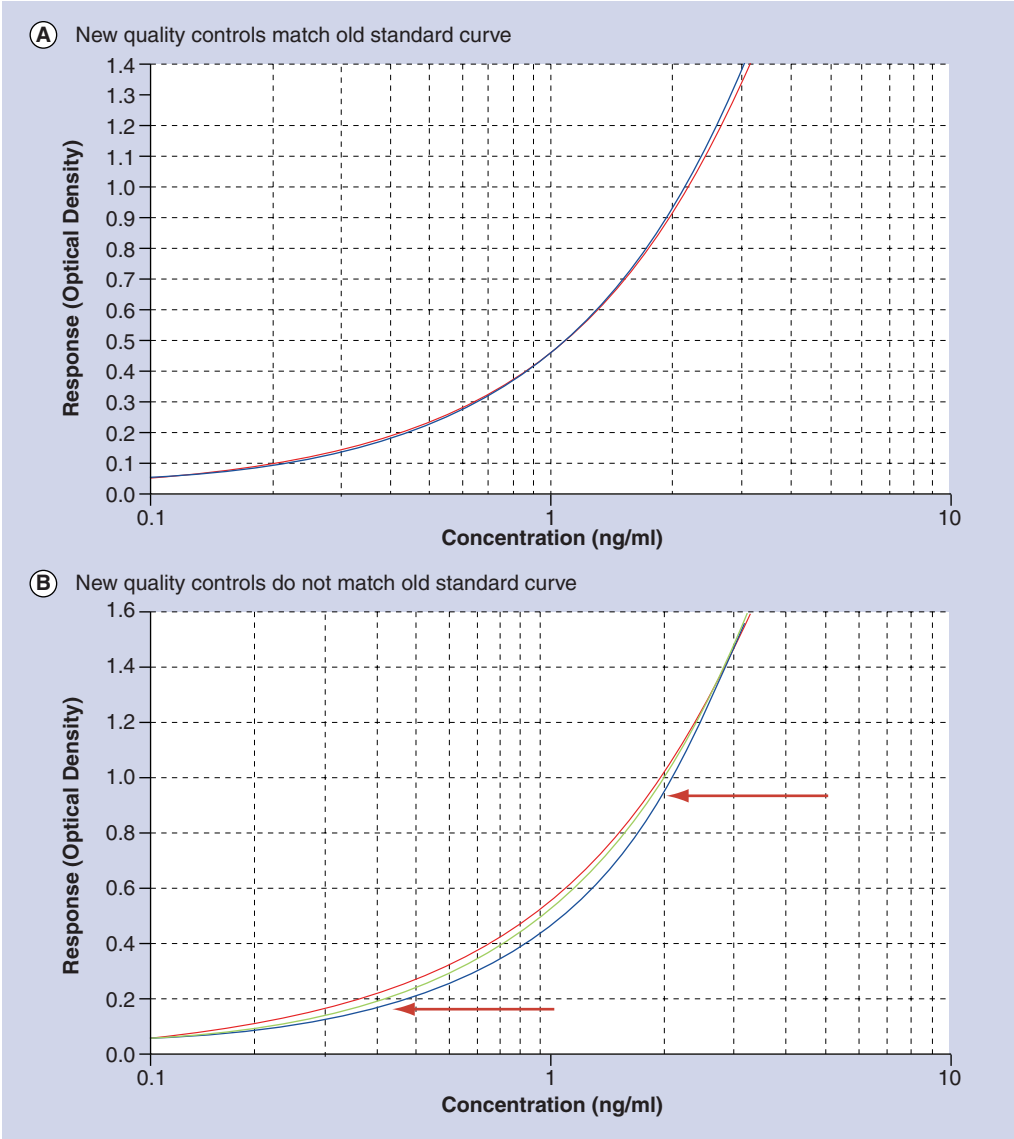

Figure 4. Standard curve comparisons. (A) Comparison of two kits whose calibrators match. **(B)** Comparison of two kits whose calibrators do not match in the middle of the analytical range (indicated by red arrows).

is no **assay drift** over time. Two considerations are, however, that some stability of the measured concentration of the endogenous sample or idea concerning the stability of the endogenous value is required and that during a prolonged study it will likely be necessary to bridge between pools of matrix. The performance of the new lot of matrix should be well characterized before the use of the old lot is discontinued.

Key Term

Assay drift: Drift or end of assay effect occurs when the performance of an assay varies across the plate due to differences in the amount of time it takes perform a manipulation.

Case study 3: bridging reference material: commercial suppliers

Another problem that has been encountered with the use of commercially available materials is the difficulty, particularly with biological reference material, in obtaining consistent commercially available reference material. Due to the length of a clinical study and the complexity of coordinating receipt of samples from multiple sites, the need for extending long-term stability frequently arises. As the acceptability of the data for the trial hinges on the long-term stability assessment, it is critical that the reference material be consistent to ensure confidence in any stability data obtained.

In the following case, samples were received by the bioanalytical laboratory over a year after they had been originally stored awaiting analysis for the 34 amino acid N-terminal fragment of PTH(1–34). The stability of analyte in matrix of

>400 days was therefore required to certify the integrity of the concentration data obtained for those samples. The example displayed in FIGURE 5 shows the successive attempts at establishing long-term stability of QC samples in matrix stored at -80°C. Stability QCs were prepared at the time of the validation with the original lot of reference material and were placed in storage at the required temperature. During the intervening period, other stability time points (3, 6 and 9 months) were tested with the original lot of reference material and passed the standard acceptance criteria; the recovered concentrations of stability QCs must be within ±20% of their nominal concentration when tested against a freshly prepared standard curve. At the time that the QCs aged to >400 days in storage, the laboratory had changed the supplier of the PTH(1–34) reference material and a second lot of reference material was purchased. It should be noted that upon receipt of the second set of reference material, the certificate of analysis of both suppliers were compared and found not to be significantly different in either the tests that were performed (e.g., purity, amino acid content) or the results obtained; these reference materials were ostensibly equivalent. Upon carrying out the long-term stability measurements however, the stored QCs did not to meet the acceptance criteria for stability evaluations (high recovery against nominal was observed). This result was confirmed with a second assay [DATA NOT SHOWN] and demonstrated not to be due to other factors (e.g., inter-analyst variability, independently prepared fresh QCs performed acceptably) and the positive bias in the recovery appeared to be inconsistent with a stability issue. A third lot of reference material was obtained from the original manufacturer, again with equivalent certificate of analysis specifications. When the stability QCs were compared with fresh calibrators prepared with this third lot of reference material the recovery of the stability QCs improved but was still outside of the acceptability range described above. After an extensive communication with the reference material provider, we were able to secure an aliquot of the original lot of reference material used for the validation and then demonstrate and confirm stability (Lot 1); the test was confirmed by running the stability.

The recommendations around this example are the following: for biologics in particular, always keeps an aliquot of the original reference material available in order to perform long-term stability; always store a sufficient number of QC samples at the appropriate temperature in case there is an issue with establishing long-term stability.

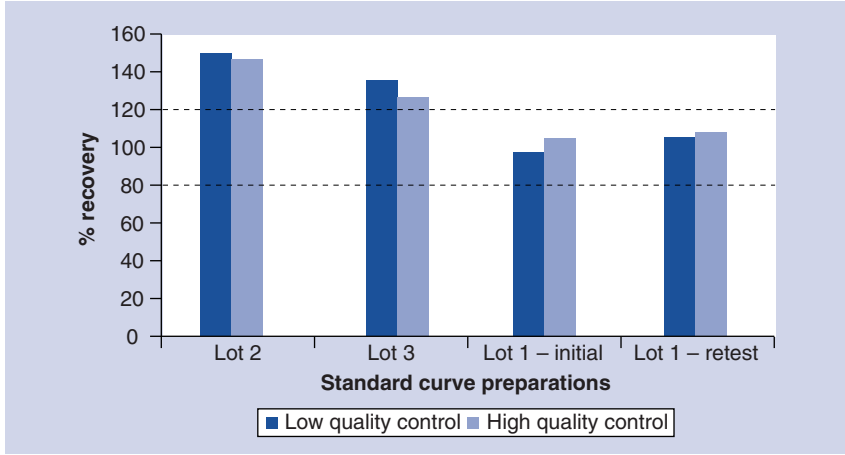

Figure 5. Comparison of stability quality controls against different lots of reference material.

Case study 4: QC of kits

The final case study relates to ensuring processes exist to make certain appropriate QCs are in place at the manufacturer. The assay in question was for a biomarker analysis used to support a set of Phase II/III clinical studies that extended over a period of 3 years. The methodology used was a sandwich ELISA format that was composed of a 96-well plate coated with an antibody that captured the biomarker of interest. Each plate was supplied as a set of 12 columnar strips of eight wells that could be removed if fewer numbers of samples were to be analyzed. The antibody was coated onto these columnar strips of wells and preloaded into the plates prior to packaging by the manufacturer.

During the validation the assay performed well, with acceptable levels of precision and accuracy, and maintained a respectable assay success rate (>95%) during the subsequent

Table 1. Quality control % nominal values.

	Quality control set A			Quality control set B		
Assay ID	LQC	MQC	HQC	LQC	MQC	HQC
Run 11	89.0	97.6	94.7	73.5†	71.2†	87.1
Run 12	88.0	95.0	96.3	71.5†	77.0†	80.0
Run 13	82.0	96.0	91.1	93.5	103.8	107.9
Run 15	97.5	99.2	99.3	60.0†	68.2†	71.7†
Run 30	94.5	94.8	100.0	72.0†	74.0†	73.3†

†Values that fell out of specification (<80%).
HQC: High quality control; LQC: Low quality control; MQC: Medium quality control.

Table 2. Plate layout of calibrators and quality controls.

	Curve 1			Curve 2			Curve 3			Curve 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD A	STD E	LQC1	STD A	STD E	LQC1	STD A	STD E	LQC1	STD A	STD E	LQC1
B	STD A	STD E	LQC1	STD A	STD E	LQC1	STD A	STD E	LQC1	STD A	STD E	LQC1
C	STD B	STD F	MQC1	STD B	STD F	MQC1	STD B	STD F	MQC1	STD B	STD F	MQC1
D	STD B	STD F	MQC1	STD B	STD F	MQC1	STD B	STD F	MQC1	STD B	STD F	MQC1
E	STD C	STD G	HQC1	STD C	STD G	HQC1	STD C	STD G	HQC1	STD C	STD G	HQC1
F	STD C	STD G	HQC1	STD C	STD G	HQC1	STD C	STD G	HQC1	STD C	STD G	HQC1
G	STD D	STD H		STD D	STD H		STD D	STD H		STD D	STD H	
H	STD D	STD H		STD D	STD H		STD D	STD H		STD D	STD H	

HQC: High quality control; LQC: Low quality control; MQC: Medium quality control.

analysis of samples. Shipments of samples were received periodically and batched for sample analysis in order to facilitate the ordering of reagents and materials. The layout of the assay plates was designed such that standards were run in the two leftmost columns with one of the set of QCs immediately adjacent to the standards. The second set of QCs was run at the end of the plate (columns on the right side of the plate), bracketing the samples that were run in the wells in the middle of the plate.

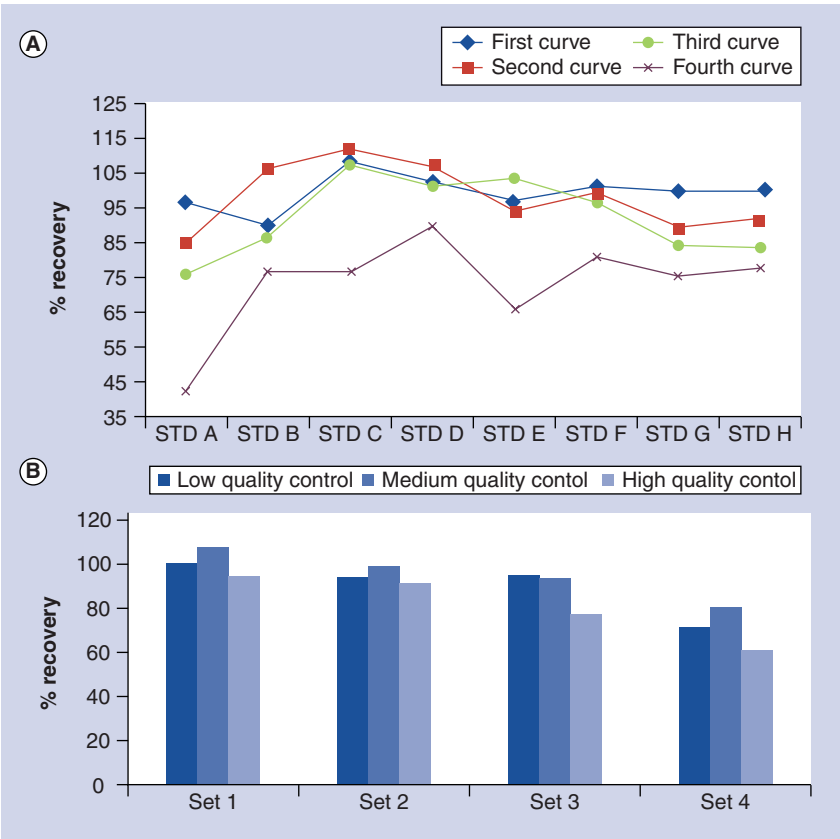

Figure 6. (A) Performance of standard curves. (B) Performance of quality controls located in different locations on the ELISA plate.

During a set of runs in the middle of one of the two concurrently running studies, the pattern of QC performance shown in Table 1 was observed. The first set of QCs on the plate, adjacent to the calibration standards, were performing well (recovery at 82–100% of the nominal concentration) while the second set of QCs at the end of the plate were consistently performing outside of the acceptable range (10/15 QCs were <80% of the nominal concentration). While these assays still met acceptance criteria according to the expectations accepted for ligand-binding assays [8–10], the clear trend in the data necessitated an investigation.

In the first set of experiments standard curves and QCs were analyzed as per the plate layout shown in Table 2. The first standard curve was used as the calibrators for all other samples and the recovery for the other standard curves is shown in Figure 6A. The performance of the first three standard curves was similar, while the fourth standard curve, the samples placed on the right side of the plate, recovered lower in comparison to the first curve on the plate. This was also reflected in the performance of the accompanying QCs (Figure 6B), with declining recoveries across the plate when compared with the standard curve in the original position (<80% for all three levels). In addition, when a plate containing one level of QC in all wells of a plate was assayed, a decline in raw responses (optical density) is also evident when the values are compared column by column (Figure 7). It was hypothesized that the declining response observed across the plate might be due to assay drift. Drift or end of assay effect is a commonly encountered issue with ELISA assays. It occurs when the performance of an assay varies across the plate, due to differences in the amount of time it takes to add samples or reagents to the entire plate from left to right or top to bottom.

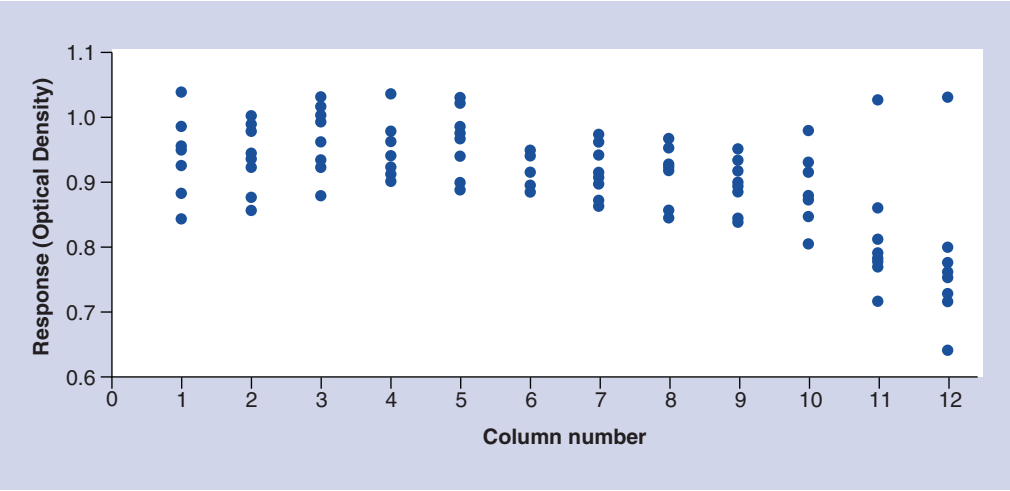

Figure 7. Performance of one level of quality control sample at different locations on the ELISA plate.

Whether assay drift could have accounted for the drop in performance between the left and the right hand side of the plate was evaluated by labeling the strips of wells, shuffling them, placing them back in the plate in a different order, and adding the samples in the same order (right to left, top to bottom). The results shown in TABLE 3 indicate the decreased performance of the assay was not due to assay drift but rather the drop in signal and performance was specifically found in the columns on the right hand side of the assay plate, as supplied by the manufacturer, as the low recovery accompanied those strips and was not related to assay drift (FIGURE 8).

The manufacturer was contacted to verify their QC processes for the release of kit lots. It was discovered that batches of plates were coated in their wells with the capture antibody and then placed in a lyophilizer to dry. The QC process consisted of randomly taking strips from different plates to make up a test plate. A single level of sample concentration was run across the plate in ten wells and the %CV on the results was calculated. The criterion for acceptance of a batch of plates was that the %CV had to be <15% on these values. Based on this criterion, it is apparent why a difference in the plate coating would not have been uncovered by this QC process. While the plates were meeting the acceptance criteria of the manufacturer, they were not performing at the same level as when the acceptance criteria for the method were set in validation.

The discovery of this issue triggered a full retrospective review of the data in order to gauge the impact on already analyzed samples.

Table 3. Quality control % nominal values and plate position.

Column	Loaded in	LQC	MQC	HQC
3	3	95.5	103.0	92.9
4	5		94.0	90.8
5	7	94.5		86.1
6	9		82.0	85.3
7	11	79.5	86.8	
8	12		91.8	79.9
9	10		78.6	76.2
10	8	69.5	70.6	
11	6		67.6	73.0
12	4	60.5	61.6	66.6

HQC: High quality control; LQC: Low quality control; MQC: Medium quality control.

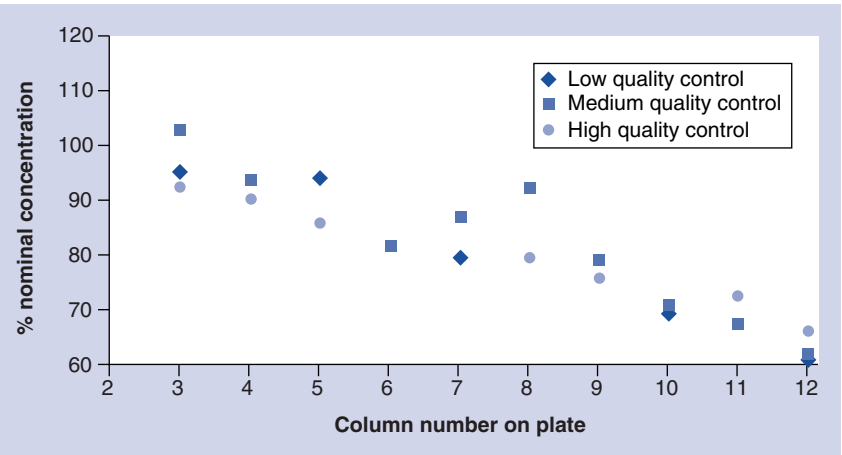

Figure 8. Recovery of low, medium and high quality controls in different columns across the ELISA plate.

Key Term

Assay validation: the process of demonstrating that analytical procedures are suitable for their intended use and that they support the measurement of the drug substances and drug products.

Those that were identified as having been run in the affected columns were reanalyzed. As the investigation into the root cause of the inconsistency revealed that the QC process at the vendor was not sufficient to keep the assay in the validated state, we approached the vendor with a request to change that process. However, the vendor of the assay kits was reluctant to change their QC process for ensuring plate-to-plate consistency when approached with the observed plate effects. Consequently, an internal QC processes had to be established for assessing the performance of incoming shipments of assay plates for the column effect, whereby a representative plate was tested from each new incoming batch.

The investigation and the implementation of the resulting corrective actions resulted in a significant (2–3 month) delay in the final database closure for the study. From the perspective of the client, eager to complete their study and meet their milestones, this had a significant impact. From the perspective of the contract research organization laboratory, the relationship with the client was damaged and the cost of the troubleshooting and internal QC process made the assay less efficient.

Conclusion

Commercially available kits are an attractive and cost-effective alternative to the occasionally arduous process of generating reagents, but offer challenges for **assay validation** and for ensuring consistency in performance throughout drug-development programs. The examples provided in this article showed that prior to embarking on programs using such kits and reagents, clear processes are required for qualifying these components prior to the use. Considerations include how different lots of reference material and kits are qualified prior to use, what procedures will be used to bridge between different lots of material (both reference material and kits) and how to ensure the consistency of kits over time (e.g., use of matrices with endogenous levels). While challenging, one should plan to perform these qualifications ahead of the arrival of clinical samples to avoid potentially costly delays. In the same way that sponsors audit and work with contract laboratories to ensure the quality of their data, laboratories should work closely with their suppliers to understand their QC processes and ensure supply for the duration of clinical studies. While this may seem evident, this step is not as simple to implement or as frequently employed. However, while unable to prevent all issues, the time and cost of performing this type of assessment is minor in comparison to the cost of performing a thorough investigation (e.g., full-time equivalents and reagents) or the delay of a clinical program.

Future perspective

The number of biological therapeutics will continue to increase in the next 5 to 10 years. Similarly, the use of biomarkers in preclinical and clinical studies will become more frequent as their number of biomarkers and our understanding of their significance becomes more sophisticated. Consequently, the use of commercially available kits and materials for their analysis will continue into the future. As assay requirements and the demand for these kits improves, pressure will continue to grow on kit manufacturers to standardize the evaluations performed prior to their commercial release to meet the needs of the bioanalytical laboratory and the drug-development process. As these criteria evolve there will be increased acceptance of the use of research-grade kits for bioanalysis.

Financial & competing interests disclosure

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.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Plan ahead when developing an assay based upon a commercially available kit.
- Identify critical reagents and pitfalls that might affect performance so that one can rapidly identify issues when the assay performance starts to fail.
- Test different suppliers and lots of reference material during qualification/validation to gain confidence in the reference material selected for validation.

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