Challenges In Using A Commercial Kit For Biomarkers Analysis

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Introduction

Well established biomarkers have been used for many years for disease diagnostics and to assess disease development and its response to treatment. Under the current mechanistic approach to drug development, biomarkers have become a prominent component of the decision making process as they can offer evidence that the intended target is hit and thus assist in making critical "go / no go" decisions on costly development steps.

The need for bioanalytical assays to support biomarkers analysis is increasing accordingly. The extent of development and validation efforts needed for these assays will depend on the intended use of the biomarker in a "fit for purpose" approach. As an example of our process for biomarker assay implementation for advanced clinical applications, we present here data from pre-study validation and in-study validation of a quantitative ELISA for measuring an established biomarker in human serum to support PK/PD studies.

Assay Development

- Normal endogenous levels for the biomarker under consideration range from 0-20pM to 60-200pM depending on physiological state, higher concentrations are observed in some diseased individuals
- The assay must be able to detect low levels of biomarker in human serum
- The assay must be performed under GLP and will be used to support submission of clinical data to regulatory bodies

A commercial kit (Table 1) based on the sandwich ELISA format (Figure 1) was selected.

Component	Description	Used in assay
96 well plate	Coated monoclonal anti-biomarker antibody,Pre-blocked	✓
Calibrators	4 levels of biomarker, in protein buffer levels are lot dependent	✗
Conjugate	Peroxydase anti-biomarker, in protein buffer	✓
Substrate	TMB solution, stabilized	✓
Stop Solution	Sulfuric Acid solution	✓
Wash Buffer	Detergent in buffered solution	In house equivalent

Table 1: Components of Biomarker Commercial ELISA Kit.

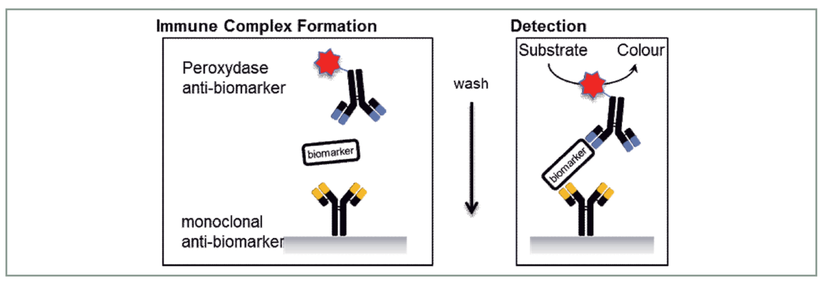

Figure 1: Assay Format.

The kit was adapted as follows:

- A biosynthetic form identical to the endogenous biomarker is available and was used as reference standard
- 8 calibrator levels were prepared using human serum affinity depleted for the endogenous biomarker from commercial source as matrix and spiked with reference standard; the 4 kit supplied calibrators were used only as an initial reference during method development
- Quality controls are not provided with the kit; Quality control samples were prepared in a human serum pool with low endogenous levels of biomarker obtained from commercial source. Biomarker levels are adjusted to final nominal QC levels by spiking with the reference standard
- Sample volume was adjusted from the original kit protocol and LLOQ was lowered to 15 pM

Matrix Pool Selection for QCs Preparation

We wanted to establish a strict procedure for matrix pool selection for QC preparation to ensure consistency during a multi-years clinical study.

A matrix pool for preparation of the study Quality controls (LQC, MQC, HQC, and DQC) must fulfil the following criteria:

- Have an endogenous biomarker level ≤ 45 pM (LQC level)
- Contain at least 5 individuals

Individual human sera from commercial sources were evaluated in the assay for endogenous biomarker levels during both assay development and in study (Figure 2). Around 1/3rd of all individual tested had levels of biomarker at or below 45 pM (22 out of 68 individuals, Figure 2).

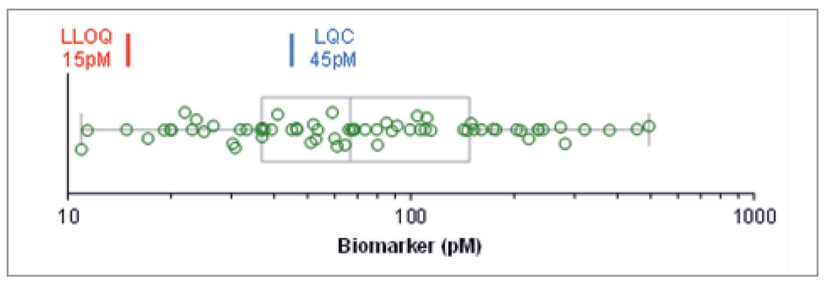

Figure 2: Endogenous biomarker level in 68 individual normal human sera from commercial sources.

A general strategy for matrix pool preparation is defined based on these results. By screening a minimum of 20 individual normal commercial sera we are typically able to find at least 5 individuals to prepare a pool.

Median biomarker concentration was 66 pM. From this data it is unlikely that a commercially prepared pool will fulfil the endogenous level criteria and a pre-screening of individuals for pool preparation is required.

Once individuals are selected, the final endogenous biomarker level is determined by measuring the resulting pool in the assay:

- The pool is measured in 3 independent runs with a minimum of 6 independent determinations per run
- The average of all determination is taken as nominal endogenous biomarker level and used to calculate the amount of reference standard to be spiked on top to reach target QC levels

Assay Performance

Pre-Study Validation

The following parameters were evaluated within assay pre-study validation based on the current guidelines for PK ligand binding assay validation.

- Calibration curve
- Precision / accuracy
- Selectivity
- Specificity
- Dilutional linearity
- Stability (freeze / thaw, short term, long term)

	LLQC 15 pM	LQC 45 pM	MQC 180 pM	HQC 340 pM	ULQC 480 pM	DQC 2000 pM
%Bias	-7.4	1.1	-0.8	1.6	8.0	6.8
%CV Inter run	0.0	0.9	3.8	0.8	0.0	1.2
%CV Intra run	10.2	3.4	2.3	4.2	7.4	3.7
Selectivity	10/10 individuals at low biomarker level			9/10 individuals at high biomarker level		
Dilutional linearity	Up to 400 fold dilution from high concentration control					

Table 2: Selected assay performance data from pre-study validation; DQC: dilution QC.

The selection of matrix for preparation of calibrators and QCs minimizes matrix interference in the assay as confirmed by the results for selectivity in individuals and dilutional linearity. Other pre-study validation results, including assay precision and accuracy, also fulfil regulatory criteria for validation of a ligand binding assay.

In-Study Monitoring

Parameters for assay performance are presented for runs carried out over an 18 months study period:

- A total of 523 runs were performed with a 92% validity rate
- 4 lots of ELISA kit were used
- 7 lots of calibrators were used, prepared from 4 lots of affinity depleted serum obtained from 2 distinct suppliers (Figure 3, panel A)
- 4 lots of pools were prepared from selected individuals with endogenous biomarker levels from 28.2 pM to 42.8 pM
- 9 lots of QCs were used (Figure 3, panel B)
- QC performance satisfied requirements for assay precision and accuracy

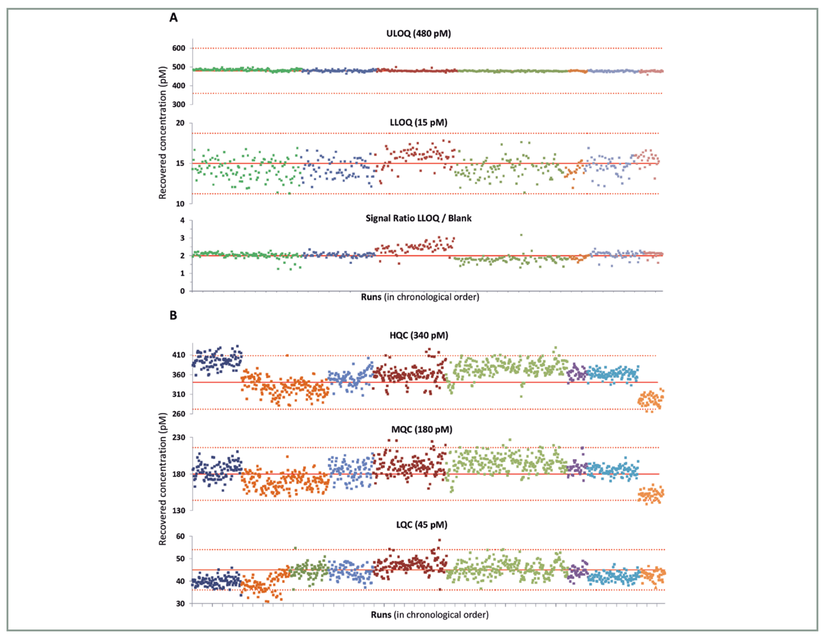

Figure 3: Reproducibility of Calibrators and QCs.

(A) Recovered biomarker concentration at LLOQ/ULOQ and signal ratio LLOQ / blank from calibrators and (B) Recovered biomarker concentration for QCs; data from 482 valid runs, each dot color represent a different lot of calibrator or QC solid line: nominal level, dotted line: nominal ± 25 % (ULOQ/LLOQ) or ± 20 % (QCs).

In-Study Monitoring of Assay Critical Reagents

The ELISA kit components used and the affinity depleted serum for preparation of calibrators were identified as assay critical reagents during method development. Bridging experiments based on QCs performance were systematically conducted for qualification of a new lot of critical reagent and to monitor lot to lot consistency.

ELISA Kit Lots

Variability between lots of ELISA kit observed in-study was negligible and did not affect assay performance.

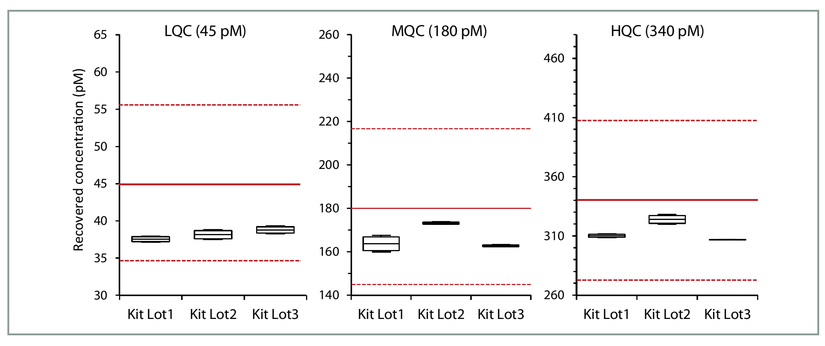

Figure 4: Lot to Lot Variability of ELISA Kit.

QCs evaluated against the same lot of calibrators in 3 of the 4 lots of ELISA kit used in the study are shown; the recovered biomarker concentration is reported, nominal QC level (solid line) ± 20 % (dotted line).

Affinity Depleted Serum Lots

Affinity depleted serum are available from multiple supplier with similar low level of endogenous biomarker as reported in their respective certificate of analysis (Table 3).

	Biomarker level	Total Protein	Stated Source	Additional processing
Supplier1 Lot1	0.5 mIU/L (3pM)	Not stated	Human Serum Normal Pooled	Not stated
Supplier1 Lot2	< 0.5 mIU/L (< 3pM)	51 g/L	Human Plasma delipidated, defibrinated	Not stated
Supplier2	0.37 mIU/L (2.2 pM)	57 g/L	Human Plasma	Filtered 0.2µm
Supplier3	< 0.5 mIU/L (< 3pM)	Not stated	Human Serum Normal Pooled	Filtered 0.2µm

Table 3: Certificate of analysis data for 4 different lots of affinity depleted serum.

Lot to Lot bridging experiment for calibrators prepared in the 4 lots of affinity depleted matrix from Table 3 are presented in Figure 5.

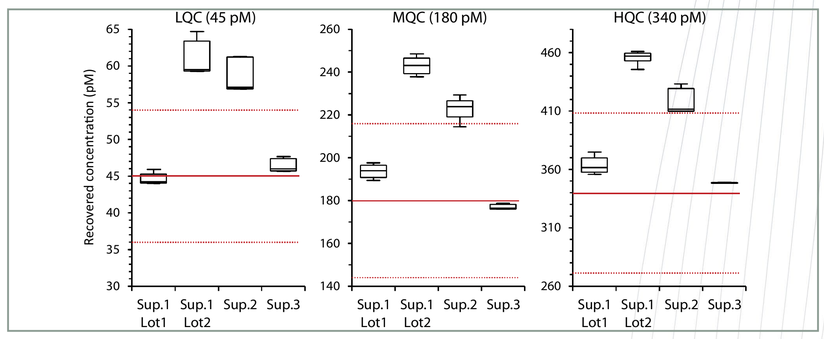

Figure 5: Lot to Lot Variability of Affinity Depleted Serum for Standard Preparation.

QCs were evaluated against calibrators prepared in 4 different lots of affinity depleted human serum obtained from 3 suppliers (Sup.1 to Sup.3); these QCs were originally evaluated against calibrators prepared in supplier1/lot1 affinity depleted serum. Recovered biomarker concentration is reported, nominal QC level (solid line) ± 20 % (dotted line).

Despite being sold under the same reference, 2 lots obtained from supplier 1 showed marked differences with Lot 2 failing QC acceptance.

Out of 2 additional suppliers, only one delivered a product that passed QC acceptance.

It is noticeable that calibrators prepared in nonperforming lot of affinity depleted serum have a lower signal than calibrators prepared in performing lots, resulting in apparent higher nominal values for the measured QCs (Figure 5). This rules out a higher than stated endogenous levels of biomarker as the cause for bias in these non performing serum lots.

From a review of the certificate of analysis it appears that the stated source is different in the non performing affinity depleted serum lots (Table 3). For such a critical reagent, it is thus essential to identify a supplier showing consistency in their qualification procedures that is reflected in the certificate of analysis provided. This also gives additional insurance about consistency of the supplier's own supply chain and thus decreases the risk of lot to lot variability. However parameters affecting performance may not be reflected in the CoA. For example presence of anti-analyte antibodies in the final product, such as traces of the capture antibody used for affinity depletion, will have a critical impact on the ability to detect the biomarker and may explain lot to lot variability. Securing as much as possible of a performing lot of critical reagent is a typical preventive measure to avoid problems with lot to lot variability but this can only be a short to midterm solution and is difficult to sustain over a multi-years clinical study.

Conclusion

The approach we conducted for kit adaptation and assay validation is consistent with the FDA draft guidance (2013) for bioanalytical method validation covering endogenous proteins and biomarkers (Table 4).

	FDA Draft Guidance (2013)	Assay
Calibrators	■ prepared in same biological matrix as study sample Free of endogenous analyte ■ minimum 6 non zero calibrators concentration	■ in human serum, affinity depleted for endogenous biomarker ■ 8 levels of biosynthetic reference standard (15-480 pM)
Quality Controls	■ prepared in same biological matrix as study sample Endogenous concentration of analyte evaluated ■ QCs prepared by spiking known quantities of analyte accounting for endogenous concentration	■ in human serum pool with defined low endogenous level of biomarker ■ QC concentration adjusted to nominal level by spiking with biosynthetic reference standard
Validation	■ "fit for purpose" approach ■ data for regulatory action need a fully validated assay using similar approach to PK assay validation	■ PK validation performed on main assay parameters

Table 4: Assay development and validation compared to draft guideline.

- In-study assay performance was in line with pre-study assay validation results and a low rate of run failure was observed
- We were able to successfully obtain several lots of low level matrix pool for QC preparation by defining procedures for (i) selection of individuals based on distribution of endogenous levels observed during method development and confirmed in-study and (ii) determining precisely and accurately biomarker endogenous levels in the resulting pools
- By performing systematic lot to lot bridging of identified critical reagents under defined procedures we were able to successfully perform the assay using different lots of ELISA kits and ensure consistent quality of calibrators

Taken together, this approach was successfully applied to validate a biomarker assay compliant with regulatory expectations and to ensure assay consistency and robustness over the time frame required for multi-years clinical studies.

Poster presentation at EBF 7th Open Meeting, November 19-21 2014

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