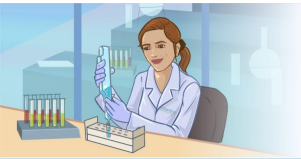

# Challenges in Biomarker method validation: A practical approach towards QC preparation and case studies

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## Introduction

Biomarker method validation has several inherent challenges viz. unavailability or poorly characterized reference standard materials, high endogenous serum or plasma level, preparation of quality control samples to name a few. Unlike PK method validation, biomarker method validation is complex since the recombinant protein used as reference standard to make QC samples is never identical with endogenous protein biomarker. Also, the protein may have purity, stability and other issues. Choosing an appropriate matrix for QC preparation remains a challenge due to high endogenous concentrations.

## Inherent biomarker Challenges

There were some unique challenges that were encountered during biomarker method validation of two molecules namely tissue factor pathway inhibitor (TFPI) and Insulin like growth factor (IGF-I). These method validations were performed as part of pharmacodynamic parameters for two different studies.

***The major challenges involved the reference standard:***

Reference standards are available as recombinant proteins which in many cases does not prove suitable for the assay. Reason being, incomplete characterization, structural anomaly from endogenous counterpart, stability issues in authentic matrix.

***Endogenous presence of the biomarker proteins in serum or plasma:***

This phenomenon makes formulation of QC samples in serum or plasma complicated, especially at lower level e.g. LLOQ-QC and LQC.

Biomarker proteins may be present in bound form in the biological fluids which requires additional treatment step for preparation of QCs. These could affect precision and accuracy of the method and may require additional work. This also might be the reason that recombinant protein does not perform well in serum or plasma QC.

## Method Validation of TFPI:

*Major Issues / Challenges encountered*

| | |
|---|---|
| **Commercial Research based kit** | Procured research based kit from reputed vendor. No quality control available in the kit or purified recombinant protein to prepare QCs in bulk. |
| **Reference Standard** | There was no innovator drug available to be used as reference standard. |
| **Preparation of Quality Controls** | QC samples prepared using recombinant human TFPI did not work in the assay. The response of QC samples was much lower when analyzed against calibration curve (with calibrators from the kit) . |

*Strategies for overcoming the challenges*

| | |
|---|---|
| **Commercial Research based kit** | The human TFPI standard provided in the kit was used to construct the calibration curve only. The same protein was not used for preparation of QC. |
| **Reference Standard** | Procured recombinant human TFPI from reputed vendor to be used as reference standard. |
| **Preparation of Quality Controls** | Quality controls at two levels were prepared in human citrated plasma. Plasma samples were screened for high and low endogenous TFPI concentration. High and low matrix (MC) i.e. plasma controls were used for precision, accuracy and stability experiments.<br>Three levels of commercial controls for TFPI spanning the calibration range were procured. These controls have been used in all validation experiments. |

## Result

**QC data obtained using recombinant human TFPI**

| QCs prepared using purified recombinant human TFPI | | | |
|---|---|---|---|
| **Sample** | **Mean OD** | **CV** | **% Error** |
| High Control-1 | 1.128 | 0.9 | -59.023 |
| High Control-2 | 1.129 | 0.8 | -58.976 |
| Low Control-1 | 0.074 | 1.2 | -56.763 |
| Low Control-2 | 0.075 | 0.8 | -55.915 |
| Mid Control-1 | 0.529 | 0.9 | -59.237 |
| Mid Control-1 | 0.52 | 5.5 | -59.935 |

The precision and accuracy of standard concentrations were measured from multiple assays performed over several days.

| Calibration Std. Conc. (pg/mL) | Mean Calculated Conc. (pg/mL) | Std. deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 2000 | 2002.341 | 8.62 | 0.4 | 0.1 | 100 | 6 |
| 1000 | 1003.49 | 8.585 | 0.9 | 0.3 | 100 | 6 |
| 500 | 492.711 | 6.272 | 1.3 | -1.5 | 98.5 | 6 |
| 250 | 251.775 | 3.096 | 1.2 | 0.7 | 101 | 6 |
| 125 | 126.402 | 0.846 | 0.7 | 1.1 | 101 | 6 |
| 62.5 | 62.439 | 1.664 | 2.7 | -0.1 | 99.9 | 6 |
| 31.25 | 31.087 | 0.807 | 2.6 | -0.5 | 99.5 | 6 |

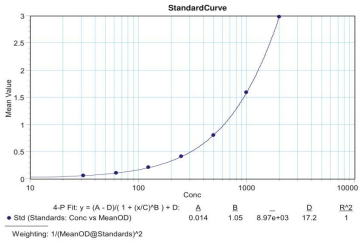


*Fig 1: Back Calculated Standard Concentrations, accuracy, precision and representative 4-PL calibration curve of TFPI [Concentration Vs Mean OD].*

### Accuracy and Precision

Intra-day accuracy and precision were determined by analyzing six replicates of QC samples at each level of concentration within a single run. Inter-day accuracy and precision were determined by analyzing six replicates of each QC sample in six independent runs on separate days.

| QC Conc. (pg/mL) | Mean Calculated Conc. (pg/mL) | Std. deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 31615.225 | 29195.316 | 391.024 | 1.3 | -7.7 | 92.3 | 6 |
| 16857.065 | 15365.43 | 351.777 | 2.3 | -8.8 | 91.2 | 6 |
| 905.159 | 903.938 | 16.339 | 1.8 | -0.1 | 99.9 | 6 |
| 482.159 | 467.238 | 10.339 | 2.3 | -3.2 | 96.8 | 6 |
| 153.206 | 157.198 | 4.368 | 2.8 | 2.6 | 103 | 6 |

*Fig 2: Intra-day precision and accuracy of QC samples.*

| QC Conc. (pg/mL) | Mean Calculated Conc. (pg/mL) | Std. deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 31615.225 | 29979.01 | 1638.503 | 5.5 | -5.2 | 94.8 | 6 |
| 16857.065 | 15761.501 | 647.062 | 4.1 | -6.5 | 93.5 | 6 |
| 905.159 | 918.204 | 35.105 | 3.8 | 1.4 | 101 | 6 |
| 482.159 | 475.748 | 16.425 | 3.5 | -1.4 | 98.6 | 6 |
| 153.206 | 158.426 | 6.495 | 4.1 | 3.4 | 103 | 6 |

*Fig 3: Inter-day precision and accuracy of QC samples.*

## Method Validation for IGF-I

*Major Issue / Challenges faced*

| | |
|---|---|
| **Serum binding protein (IGFBP)** | IGF-I binds to IGF-I binding protein (IGFBP) in serumto form a ternary complex. This complex greatly stabilizes IGF in the circulation. Due to this binding, it requires additional strategies to make IGF-I free to be detected in the assay. |
| **Reference standard** | Recombinant human IGF-I (rhIGF-I) was procured from a reputed vendor. The protein failed to behave in serum as the endogenous IGF-I in the assay |
| **Preparation of Quality Controls** | QC samples were prepared in human serum by fortifying rhIGF-I. Freshly prepared serum QC samples at all levels met acceptance criteria.<br>This serum QC samples when stored in freezer & analyzed after few days, the recovery found was very low compared to fresh QCs. This was tested multiple times and same results obtained.<br>This may be because of heterogeneity of rhIGF-I from endogenous counterpart. |

*Strategies for overcoming the challenges*

| | |
|---|---|
| **Serum binding protein (IGFBP)** | To release IGF-I from bound complex, an acid pretreatment followed by neutralization was used. |
| **Reference standard** | rhIGF-I was used in preparing surrogate QCs which were prepared in diluted human serum. The rhIGF-Iperformed well when prepared in 10% serum. |
| **Preparation of Quality Controls** | Quality controls at three levels were prepared in human serum. Serum samples were screened for high, medium and low endogenous IGF-I concentration. High, medium and low matrix (MC) i.e. serum controls were used for precision, accuracy, stability and all other validation experiments.<br>Additionally, QC samples at two levels were prepared in 10% serum by fortifing rhIGF-I. These QCs fall at high and low end of the curve. |

The precision and accuracy of standard concentrations were measured from multiple assays performed over several days.

| Calibration Std. Conc. (ng/mL) | Mean Calculated Conc. (ng/mL) | Std. deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 6 | 6.47 | 0.118 | 1.8 | 7.8 | 108 | 6 |
| 3 | 3.187 | 0.071 | 2.2 | 6.2 | 106 | 6 |
| 1.5 | 1.404 | 0.071 | 5.1 | -6.4 | 93.6 | 6 |
| 0.75 | 0.687 | 0.009 | 1.3 | -8.4 | 91.6 | 6 |
| 0.375 | 0.342 | 0.014 | 4.1 | -8.8 | 91.2 | 6 |
| 0.188 | 0.182 | 0.006 | 3.3 | -3.2 | 96.8 | 6 |
| 0.094 | 0.109 | 0.004 | 3.7 | 16 | 116 | 6 |

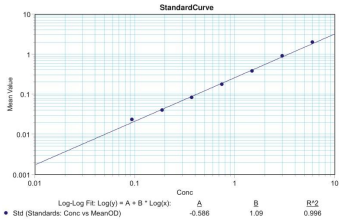


*Fig 4: Back Calculated Standard Concentrations, accuracy, precision and representative Log-Log Fit calibration curve of IGF-I [Concentration Vs Mean OD].*

### Accuracy and Precision

**Intra-assay**

| QC Conc. (ng/mL) | Mean Calculated Conc. (ng/mL) | Std. Deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 186.387 | 179.993 | 5.695 | 3.2 | -3.4 | 96.6 | 1 |
| 100.637 | 100.259 | 3.499 | 3.5 | -0.4 | 99.6 | 1 |
| 48.375 | 41.511 | 2.836 | 6.8 | -14.2 | 85.8 | 1 |
| 450 | 504.6 | 0.304 | 6 | 12.1 | 112 | 1 |
| 28 | 280 | 0.008 | 2.9 | 0 | 100 | 1 |

*Fig 5: Intra-day precision & accuracy of QC samples*

**Inter-assay**

| QC Conc. (ng/mL) | Mean Calculated Conc. (ng/mL) | Std. Deviation | % CV | % Bias | Mean Accuracy | n |
|---|---|---|---|---|---|---|
| 186.387 | 181.727 | 6.234 | 3.4 | -2.5 | 97.5 | 4 |
| 100.637 | 100.971 | 3.366 | 3.3 | 0.3 | 100 | 4 |
| 48.375 | 42.748 | 2.466 | 5.8 | -11.6 | 88.4 | 4 |
| 450 | 510.4 | 0.269 | 5.3 | 13.4 | 113 | 4 |
| 28 | 286.0 | 0.009 | 3.1 | 2.1 | 102 | 4 |

*Fig 6: Intra-day precision & accuracy of QC samples*

**Note: Most biomarkers are coverd under CAP Proficiency testing Program (EQA program for relative accuracy)**

## Conclusion

***Due to various heterogeneous forms of proteins, preparation of QCs for biomarkers remains a challenge. Assaying matrix controls along with QCs in surrogate matrix remains one of the many options. A 'fit to purpose' strategy may be the approach in biomarker validation for overcoming its challenges. Guidances and guidelines continue to grow and evolve for better clarity.***