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PARALLELISM CHALLENGES WHEN USING COMMERCIAL KITS FOR BIOMARKER QUANTITATION

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PURPOSE

Parallelism assessments are crucial to biomarker method development and validation. Endogenous biomarkers are compounds that originate from within an organism. Parallelism in human or animal matrices is evaluated using endogenous samples, diluted multiple times to cover the quantitative range of the calibration curve. Parallelism assessments give information on many different aspects of the method – most notably, they determine the validity of proxy matrices and help establish the MRD.

Biomarkers are mostly quantified using commercially available kits with recombinant reference standards. Therefore, it is important to ensure that the endogenous compound and the reference standard are immunologically similar. Here we describe challenges and mitigation strategies observed for three different biomarkers and demonstrate that different approaches are required for each one.

OBJECTIVE(S)

In this study, three parallelism issues were addressed:

1. Impact of the albumin reference standard on parallelism using a colorimetric kit as per manufacturer recommendations
2. Impact of proxy matrices on parallelism for the biomarker Surfactant Protein-D analyzed using an ELISA kit
3. Impact of ELISA kits from different suppliers on parallelism results for Serum Amyloid A (SAA)

METHODS

Albumin was quantified using the bromocresol purple (BCP) albumin assay kit. BCP forms a colored complex specifically with albumin and the intensity of the color is measured at 610 nm.

SP-D and SAA ELISA kits are based on the general principles of the sandwich ELISA and follow largely the same protocol. The standard curve for each method was prepared in a proxy matrix by spiking the reference standard in a kit provided diluent. The manufacturer's protocol was followed for each ELISA. The absorbances and known standard curve concentrations were plotted and used to calculate the target protein concentrations in the parallelism samples.

Human serum or plasma samples were serially diluted in kit-specific buffer to achieve the sample dilutions. Dilutions tested were selected to span a significant portion of the concentration range covered by each kit's standard curve, based on advice from the kit manufacturer regarding the expected concentration of the target analyte in serum. The dilution-adjusted concentrations were calculated by multiplying the dilution factor with the measured concentration. The recovery between sample dilutions was determined by dividing the dilution-adjusted concentration at each dilution by the measured concentration of the reference dilution. The one that was most common, and showed a passing relative recovery rate at more than one dilution, was selected as the minimum required dilution (MRD).

RESULTS

1. Albumin

Objective: Determination of albumin in clinical samples for the quantitation of testosterone

Figure 1. Calibration Curve of Albumin Using Kit-Supplied Reference Standard

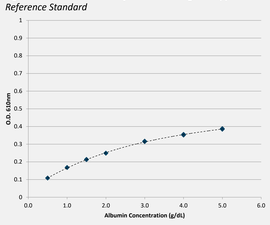


Table 1. Dilution-Adjusted Concentrations of Albumin in Four Human Serum Lots

Dilution Factor	Albumin Conc. (g/dL)			
	Human serum A	Human Serum B	Human serum C	Human Serum D
2X	>ULOQ	>ULOQ	8.48	8.76
3X	8.64	>ULOQ	NA	NA
4X	6.29	8.40	NA	NA
5X	5.42	7.20	5.60	6.0
7X	5.26	6.15	NA	NA
10X	4.55	6.45	6.60	6.80
%C.V.	**26.3**	**14.2**	**21.2**	**19.8**

- OD at ULOQ = 0.4
- Parallelism acceptance criteria: CV ≤ 30.0%
- Acceptable CV for all lots: 14.2% to 26.3%, but trend is observed
- Gradual increase of concentration with increasing dilution factors

Figure 2. Calibration Curve of Albumin Using Human Reference Standard

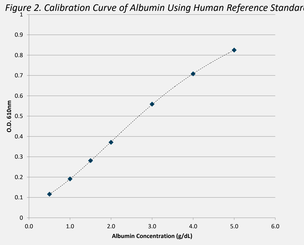


Table 2. Dilution-Adjusted Concentrations of Albumin in Four Human Serum Lots

Dilution Factor	Albumin Conc. (g/dL)			
	Human serum A	Human Serum B	Human serum C	Human Serum D
2X	4.59	5.99	5.38	5.36
4X	4.36	6.72	5.36	5.48
5X	5.24	6.28	5.35	5.30
6X	4.41	6.93	5.28	5.64
7X	4.90	5.43	4.90	5.53
%C.V.	**7.8**	**9.5**	**3.8**	**2.5**

- External reference standard (human)
- OD at ULOQ = 0.8
  - Increase of 2X
- Parallelism CV = 2.5% to 9.5%
  - <10.0% for all lots
- Precision (CV) increased by up to 18%

ALBUMIN CONCLUSION

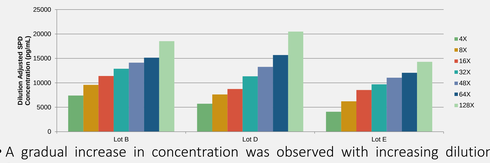
The parallelism issue observed with the BCP kit was resolved by changing the reference material to a source that best represents the endogenous protein. It is critical that the quality and source of the reference material is verified at the early stages of method feasibility through parallelism assessment.

2. SPD

Objective: Determination of SPD for an exploratory endpoint

- Kit-supplied diluent used

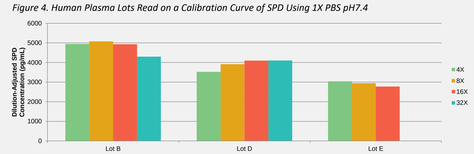
Figure 3. Human Plasma Lots Read on a Calibration Curve of SPD Using Kit-Supplied Diluent



- A gradual increase in concentration was observed with increasing dilution factors.
- CV of all three lots ranged from 29.0% to 43.2%.
- Recovery of diluted samples vs. MRD samples ranged from 130% to 350%.
- Non-specific binding interference is suspected.

Mitigation Strategy: Diluent changed to 1X PBS pH 7.4

Figure 4. Human Plasma Lots Read on a Calibration Curve of SPD Using 1X PBS pH7.4



- Dilution-adjusted concentration for all three lots was acceptable with a CV range of 4.6% to 7.2%.
- Recovery of diluted samples vs MRD samples was within the 80% to 120% range.

Table 3. Back-Calculated, Dilution-Adjusted Concentrations of SP-D Diluted in Kit Diluent or 1XPBS pH7.4

Human Plasma Lot	Dilution Factor	Kit Diluent			1XPBS pH 7.4		
		Dilution Adjusted Conc. (pg/mL)	%Recovery vs 4X	%C.V.	Dilution Adjusted Conc. (pg/mL)	%Recovery vs 4X	%C.V.
B	4 (Kit MRD)	7400	100.0	29.0	4946	NA	7.2
	8	9574	129.4		5078	102.7	
	16	11392	153.9		4931	99.7	
	32	12886	174.1		4301	87.0	
	48	14131	191.0		<LLOQ	NA	
	64	15142	204.6		<LLOQ	NA	
	128	18522	250.3		<LLOQ	NA	
D	4	5729	100.0	43.2	3528	NA	6.9
	8	7621	133.0		3914	111.0	
	16	8728	152.3		4098	104.7	
	32	11331	197.8		4106	116.5	
	48	13262	231.5		<LLOQ	NA	
	64	15686	273.8		<LLOQ	NA	
	128	20480	357.5		<LLOQ	NA	
E	4	4085	100.0	37.0	3039	NA	4.6
	8	6218	152.2		2945	96.9	
	16	8533	208.9		2773	91.2	
	32	9686	237.1		<LLOQ	NA	
	48	11045	270.4		<LLOQ	NA	
	64	12045	294.9		<LLOQ	NA	
	128	14298	350.0		<LLOQ	NA	

SPD CONCLUSION

Our SPD case study demonstrates that parallelism issues can be mitigated by changing the diluent to remove the matrix effect. The diluent components provided with a kit are not always available, therefore multiple diluents with different properties can be evaluated to remove specific or non-specific binding interference.

3. SAA

Objective: Determination of SAA for an exploratory endpoint

- SAA parallelism was assessed using three different kits.
- Kits 1 and 2 were assessed with two and six human plasma lots respectively, serially diluted up to 16X.

Table 4. SAA Concentration of Two Human Serum Lots with Kit 1

Dilution Factor (200x MRD included)	Dilution Adjusted SAA Conc. (ng/mL)	
	Lot C	Lot F
200 (MRD)	37516	51775
400	31901	46092
800	26894	39504
1600	22452	32096
3200	19488	26176
%C.V.	**26.2**	**26.4**
%Recovery (3200X vs MRD)	**52**	**50**

Table 5. SAA Concentration of Six Human Serum Lots with Kit 2

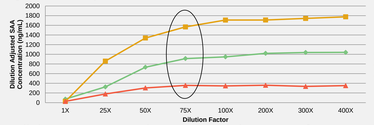
Dilution Factor	Dilution Adjusted SAA Conc. (ng/mL)					
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
1	89.2	212.8	67.8	69.2	97.0	59.9
2	70.4	NA	90.0	76.1	118.8	90.4
4	128.8	232.8	173.0	150.3	219.6	151.0
8	219.4	439.1	356.4	297.8	371.7	270.2
16	347.7	696.5	1011.5	401.6	703.8	511.5
%C.V.	**66**	**57**	**116**	**73**	**83**	**85**
%Recovery (16X vs 1X)	**494**	**327**	**1491**	**580**	**725**	**854**

- Kit 1: Gradual decrease in response with increasing dilution factors
  - Up to 50% concentration loss
- Kit 2: Up to 15-fold increase in concentration with increasing dilutions
- Kit 3: No mention of MRD in kit protocol
  - Required to establish the MRD through parallelism assessment

Table 6. SAA Concentration of Three Human Plasma Lots with Kit 3

Human Plasma Lot	Dilution Factor	Diluted Adjusted SAA Conc. (ng/mL)	%Recovery							
			1X	25X	50X	75X	100X	200X	300X	400X
B	1X	73.2	100.0	22.5	10.0	8.0	7.7	7.2	7.1	7.0
	25X	328	446.6	100.0	44.5	35.7	34.4	32.0	31.4	31.3
	50X	733	1004.1	224.8	100.0	80.4	77.4	71.9	70.6	70.5
	75X	912	1249.3	279.8	124.4	100.0	96.3	89.4	87.9	87.7
	100X	947	1297.3	290.5	129.2	103.8	100.0	92.8	91.2	91.1
	200X	1020	1397.3	312.9	139.2	111.8	107.7	100.0	98.3	98.1
	300X	1038	1421.9	318.4	141.6	113.8	109.6	101.8	100.0	99.8
	400X	1040	1424.7	319.0	141.9	114.0	109.8	102.0	100.2	100.0
C	1X	25.1	100.0	2.9	1.9	1.6	1.5	1.5	1.4	1.4
	25X	860	3425.3	100.0	64.3	54.9	50.3	50.3	49.3	49.4
	50X	1338	5328.7	155.5	100.0	85.4	78.3	78.2	76.7	75.3
	75X	1567	6242.0	182.2	117.1	100.0	91.7	91.6	89.9	88.2
	100X	1709	6808.8	198.7	127.7	109.1	100.0	99.9	98.0	96.2
	200X	1710	6812.7	198.8	127.8	109.1	100.1	100.0	98.1	96.3
	300X	1743	6944.2	202.7	130.3	111.2	102.0	101.9	100.0	98.1
	400X	1776	7075.7	206.5	132.7	113.3	103.9	103.9	101.9	100.0
E	1X	28.5	100.0	14.8	8.7	7.4	7.6	7.3	7.9	7.5
	25X	179	678.4	100.1	59.0	50.2	51.8	49.8	53.3	50.9
	50X	304	1147.2	169.8	100.0	85.2	87.9	84.4	90.5	86.4
	75X	357	1347.2	199.4	117.4	100.0	103.2	99.2	106.3	101.4
	100X	346	1305.7	193.3	113.8	96.9	100.0	96.1	103.0	98.3
	200X	360	1358.5	201.1	118.4	100.8	104.0	100.0	107.1	102.3
	300X	336	1267.9	187.7	110.5	94.1	97.1	93.3	100.0	95.5
	400X	352	1328.3	196.6	115.8	98.6	101.7	97.8	104.8	100.0

Figure 5. Dilution-Adjusted SAA Concentration vs. Dilution Factor



- Parallelism assessment demonstrated that MRD of 75X is required.

SAA CONCLUSION

Parallelism assessment can be used as a kit-selecting tool when evaluating different suppliers. Parallelism of SAA was evaluated with three different kits, and only one had acceptable recovery. Cost permitting, different suppliers should be compared in order to select the best kit available.

CONCLUSION(S)

Various approaches were used to solve parallelism issues for three different biomarkers using commercially available kits. These approaches demonstrate that a "one-size-fits-all" solution does not apply to solve all of them. Instead, scientific judgment and a fit-for-purpose method are required. Here we provide different test cases demonstrating different possible approaches to mitigate parallelism issues. Not all kit components are adequate to perform biomarker analysis with acceptable parallelism. A step-wise approach is used to address these issues. First, the source of the reference standard material needs to be confirmed and replaced, if required, to best mimic the endogenous samples. Second, the diluent buffers can be altered to remove any potential matrix effects. Third, different kits must be compared to evaluate parallelism and standard curve performance.

FUNDING / GRANTS / ENCORE / REFERENCE OR OTHER USE

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