Leveraging Parallelism Assays to Validate the Accurate Detection of Endogenous Levels of Biomarkers in Ligand-Binding Assays

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Introduction

The classical purpose of the parallelism assay is to demonstrate that the sample-dilution response curve aligns with the standard-calibrator response curve. This alignment confirms that the calibrator material is suitable for measuring the endogenous analyte. Beyond its essential role, parallelism informs several other crucial assay parameters for biomarker ligand binding assays:

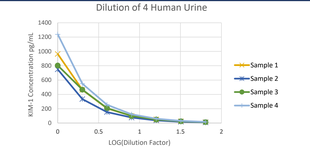
- Minimum Required Dilution (MRD): Parallelism aids in establishing the dilution level at which accurate measurements of endogenous biomarkers can be made.
- Determining the dilution factors to eliminate the matrix effect.
- Precise quantification of endogenous biomarker levels across various dilution factors.
- Lower Limit of Quantification (LLOQ): Parallelism informs the determination of the lowest concentration of the endogenous biomarkers that can be reliably quantified.

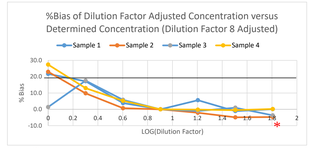
In this practical exploration of the parallelism assay, various unique biomarkers and matrices were meticulously evaluated. The parallelism assay was utilized to determine the endogenous levels of the biomarkers.

Materials & Methods

- The accuracy, precision, and parallelism assays were conducted using the sandwich immunoassay method. The capture antibody for the analytes is pre-coated onto 96-well plates and analytes present in the standards, quality controls, and test samples will bind to the capture antibody. After washing away the unbound material, a detection antibody (Sulfo-Tag conjugates) specific to these analytes is added to the wells and incubated for immunocomplex formation. After washing the plates, the read buffer is added to the wells, and electrochemiluminescence signal is read on a Meso QuickPlex SQ120mm.
- Three or four lots of human matrix with varying endogenous levels of biomarkers were tested. Initially, the samples were assessed undiluted. Subsequently, they underwent a series of dilutions to reach the Lower Limit of Quantification (LLOQ) level.
- When determining the concentration of the endogenous biomarkers, select the Minimum Required Dilution (MRD) that, when used for back-calculation and recovery rate calculation, exhibits the best overall recovery rate across all tested samples.
- The % recovery is calculated by dividing the measured actual concentration by the determined concentration through the MRD and then multiplying by 100.
- $\%\ Recovery = \frac{meaured\ concentration}{determined\ concentration} * 100$
- The percentage bias is calculated by subtracting the determined concentration obtained through MRD from the measured actual concentration, and this result is then divided by the determined concentration and multiplied by 100. The % bias should be within the ±20%.
- $\%\ bias = \frac{meaured\ concentration - determined\ concentration}{determined\ concentration} * 100$

Results



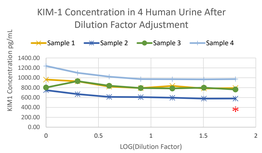


*The concentration reaches the LLOQ

Fold Dilution	Individual -1		Individual -2		Individual -3		Individual -4	
	Average % Recovery	Con. No DF Adjusted (pg/mL)	Average % Recovery	Con. No DF Adjusted (pg/mL)	Average% Recovery	Con. No DF Adjusted (pg/mL)	Average % Recovery	Con. No DF Adjusted (pg/mL)
1	122	*963*	123	*748*	101	*803*	127	*1238*
2	117	*463*	110	*334*	118	*466*	113	*549*
4	104	*205*	101	*153*	106	*210*	105	*256*
8	100	*98.9*	100	*76.0*	100	*99.1*	100	*121*
16	106	*52.2*	97.9	*37.2*	99.0	*49.0*	99.8	*60.7*
32	98.9	*24.4*	95.1	*18.0*	101	*25.0*	99.5	*30.2*
64 #	100	*12.3*	95.3	*9.06*	96.3	*11.9*	100	*15.2*

Table 2 Parallelism % Recovery for KIM-1; DF: Dilution Factor
The concentration reaches the LLOQ

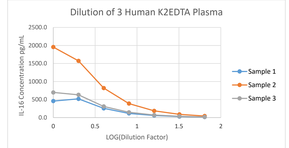
Assay for Kidney Injury Molecule -1 (KIM-1)

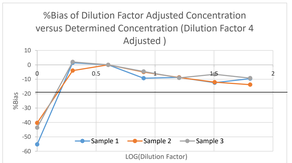


LOG(Dilution Factor)	
0	1
0.3	2
0.6	4
0.9	8
1.2	16
1.51	32
1.81	64

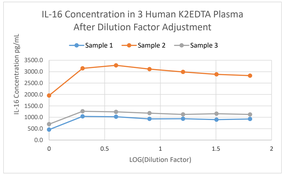
KIM-1	Nominal Conc.	Replicates per run	Run - 1		Run - 2		Run - 3	
			%CV	%Bias	%CV	%Bias	%CV	%Bias
ULOQ	20,000	6	2.11	-15.9	4.47	3.11	4.83	6.89
HQC	16,000	6	2.64	-13.0	3.88	1.14	1.58	5.77
MQC	623	6	2.06	-14.1	4.84	-5.06	2.38	3.47
LQC	58.5	6	2.92	-17.3	3.92	-6.51	2.24	2.33
LLOQ	19.5	6	5.88	-18.2	2.03	-7.82	4.01	2.35

Table 1 Assay Accuracy and Precision for KIM-1





Assay for IL-16



LOG(Dilution Factor)	
0	1
0.3	2
0.6	4
0.9	8
1.2	16
1.51	32
1.81	64

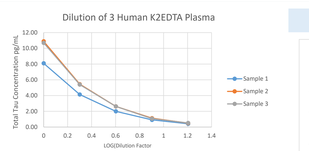
IL-16	Nominal Conc.	Replicates per run	Run - 1		Run - 2		Run - 3	
			%CV	%Bias	%CV	%Bias	%CV	%Bias
ULOQ	1,658	6	6.24	2.74	2.47	-1.48	2.31	2.04
HQC	1,327	6	3.82	5.89	3.90	3.74	2.43	2.66
MQC	82.8	6	4.08	-1.15	2.54	-4.88	1.70	-6.37
LQC	12.4	6	3.53	2.42	2.76	-5.71	3.13	-5.43
LLOQ	4.13	6	2.25	2.23	3.70	-5.16	5.72	-0.75

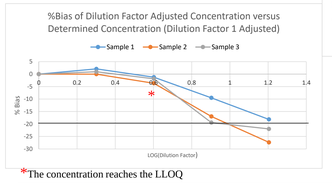
Table 3 Assay Accuracy and Precision for IL-16

Fold Dilution	Individual -1		Individual -2		Individual -3	
	Average %Recovery	Con. No DF Adjusted (pg/mL)	Average % Recovery	Con. No DF Adjusted (pg/mL)	Average % Recovery	Con. No DF Adjusted (pg/mL)
1	44.8	*459*	59.7	*1956*	56.4	*698*
2	102	*520*	96.0	*1573*	102	*632*
4	100	*256*	100	*819*	100	*310*
8	90.7	*116*	95.0	*389*	95.4	*148*
16	91.3	*58.4*	91.2	*187*	90.9	*70.4*
32	87.6	*28.0*	88.0	*90.1*	93.4	*36.2*
64	90.4	*14.4*	86.3	*44.4*	90.8	*17.4*

Table 4 Parallelism % Recovery for IL-16; DF: Dilution Factor
Samples were not diluted to the LLOQ level

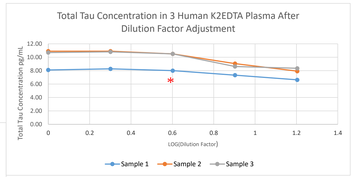
Results





*The concentration reaches the LLOQ

Assay for Total Tau

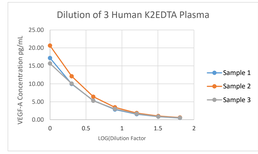


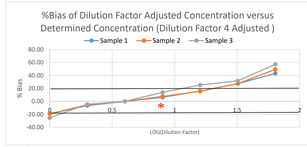
Total Tau	Nominal Conc.	Replicates per run	Run - 1		Run - 2		Run - 3	
			%CV	%Bias	%CV	%Bias	%CV	%Bias
ULOQ	2,500	6	3.70	-3.20	5.90	-8.80	2.70	-2.40
HQC	2,000	6	2.80	1.00	2.90	-4.50	1.70	2.00
MQC	400	6	2.50	1.50	2.00	-5.80	2.90	2.50
LQC	7.50	6	3.80	-8.10	3.40	-7.50	2.50	-2.10
LLOQ	2.50	6	4.00	-5.20	8.10	-1.60	2.40	-9.20

Table 5 Assay Accuracy and Precision for Total Tau

Fold Dilution	Individual -1		Individual -2		Individual -3	
	Average %Recovery	Con. No DF Adjusted (pg/mL)	Average %Recovery	Con. No DF Adjusted (pg/mL)	Average %Recovery	Con. No DF Adjusted (pg/mL)
1	100.0	*8.10*	100	*10.9*	100	*10.7*
2	102	*4.14*	100	*5.45*	101	*5.40*
4 #	98.8	*2.00*	96.3	*2.63*	98.1	*2.63*
8	90.5	*0.92*	83.0	*1.13*	80.6	*1.08*
16	81.9	*0.41*	72.7	*0.50*	78.0	*0.52*

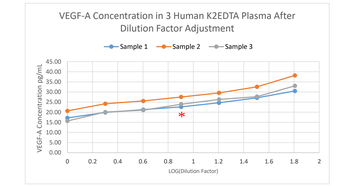
Table 6 Parallelism % Recovery for Total Tau; DF: Dilution Factor
The concentration reaches the LLOQ





*The concentration is around the LLOQ

Assay for VEGF-A



VEGF-A	Nominal Conc.	Replicates per run	Run - 1		Run - 2		Run - 3	
			%CV	%Bias	%CV	%Bias	%CV	%Bias
ULOQ	810	6	2.32	7.56	2.45	-3.48	1.57	3.28
HQC	648	6	1.95	5.21	2.73	-4.84	3.02	6.40
MQC	40.4	6	3.26	-7.17	2.08	-18.97	1.57	-7.21
LQC	6.06	6	4.05	-1.36	5.16	-16.35	3.99	-5.64
LLOQ	2.02	6	3.29	10.76	4.92	-10.94	3.52	0.61

Table 7 Assay Accuracy and Precision for VEGF-A

Fold Dilution	Individual -1		Individual -2		Individual -3	
	Average %Recovery	Con. No DF Adjusted (pg/mL)	Average %Recovery	Con. No DF Adjusted (pg/mL)	Average %Recovery	Con. No DF Adjusted (pg/mL)
1	80.7	*17.2*	80.7	*20.6*	74.5	*15.7*
2	93.5	*9.95*	94.7	*12.1*	95.5	*10.0*
4	100	*5.32*	100	*6.39*	100	*5.25*
8 #	106	*2.83*	108	*3.44*	114	*2.99*
16	116	*1.54*	116	*1.85*	125	*1.64*
32	127	*0.85*	127	*1.02*	132	*0.87*
64	143	*0.48*	149	*0.60*	157	*0.52*

Table 8 Parallelism % Recovery for VEGF-A; DF: Dilution Factor
The concentration is around the LLOQ

Results

- For endogenous **KIM-1**, the signal decreased proportionally with dilution from dilution factor 2 to the LLOQ level. Dilution factor **8** was selected as the MRD (Minimum Required Dilution) to determine the concentration.
- For endogenous **IL-16**, the concentration decreased proportionally with dilution from dilution factor 2. Dilution factor **4** was selected as MRD to determine the concentration. The samples were diluted to the 2 times of LLOQ level, not to the LLOQ level due to material limitation.
- For endogenous **total Tau**, the concentration decreased proportionally with dilution from dilution factor 1 to the LLOQ level. Dilution factor **1** was selected as the MRD to determine the concentration.
- For endogenous **VEGF-A**, the concentration decreased proportionally with dilution from dilution factor 2 to the LLOQ level. Dilution factor **4** was selected as the MRD to determine the concentration.
- For endogenous **KIM-1**, **total Tau**, and **VEGF-A**, when diluted to the **LLOQ** (Lower Limit of Quantification) level, the measured concentration, adjusted by the dilution factor, **matched** the determined concentration across the multiple samples (%bias was within ±20%).
- The measured concentrations of **KIM-1, IL-16, total Tau**, and **VEGF-A**, adjusted by the dilution factor, consistently matched the determined concentrations across multiple samples tested at varying dilution factors (with %bias within ±20%). **This confirmation validates that the determined concentrations represent the endogenous levels of these biomarkers.**

Conclusion

- Parallelism establishes the MRD dilution level at which **accurate measurements** of endogenous biomarkers can be made.
- Parallelism assay can confirm the **precise quantification** of endogenous biomarker levels across various dilution factors.
- Parallelism can be utilized in determining the dilution factors to eliminate the **matrix effect**.
- Parallelism informs the determination of the **lowest concentration** of the endogenous biomarkers that can be reliably quantified.
- Parallelism should be evaluated in a ligand-binding assay when samples are available at concentrations 4-8 times than the Lower Limit of Quantification (LLOQ) or higher.

References

1. Tu, J., & Bennett, P. (2017). Parallelism experiments to evaluate matrix effects, selectivity and sensitivity in ligand-binding assay method development: pros and cons. Bioanalysis, 9(14), 1107–1122.
2. Nelson, R. (2016). TT-61: Non-parallelism in biomarker assays. Presented on behalf of EBF TT-61 at the EBF Open Symposium, November 2016, Barcelona, Spain

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