Low Cut Points: Where Has Our Biological Variability Gone?

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INTRODUCTION

Advancements in technology have considerably mitigated non-specific binding in anti-drug antibody (ADA) assays, resulting in decreased assay background and increased sensitivity. Consequently, newly developed ADA assays frequently exhibit very low assay cut points (CPs) and sensitivities. Low CPs are appropriate if they reflect the inherent inter-individual variability within patient populations. But what if the assay fails to detect biological variability? In such cases, do low CPs truly represent the population or do they merely reflect the instrument detection limit?

Here we present an assay characterized by low CP and assay background, and the effort to verify the suitability of the assay.

METHOD

The assay described in this case study is a standard bridging electrochemiluminescent immunoassay detecting ADAs against a bispecific therapeutic antibody ranked with moderate immunogenicity risk. Investigations into domain specificity were conducted through the inclusion of characterization assays A and B (Figure 1).

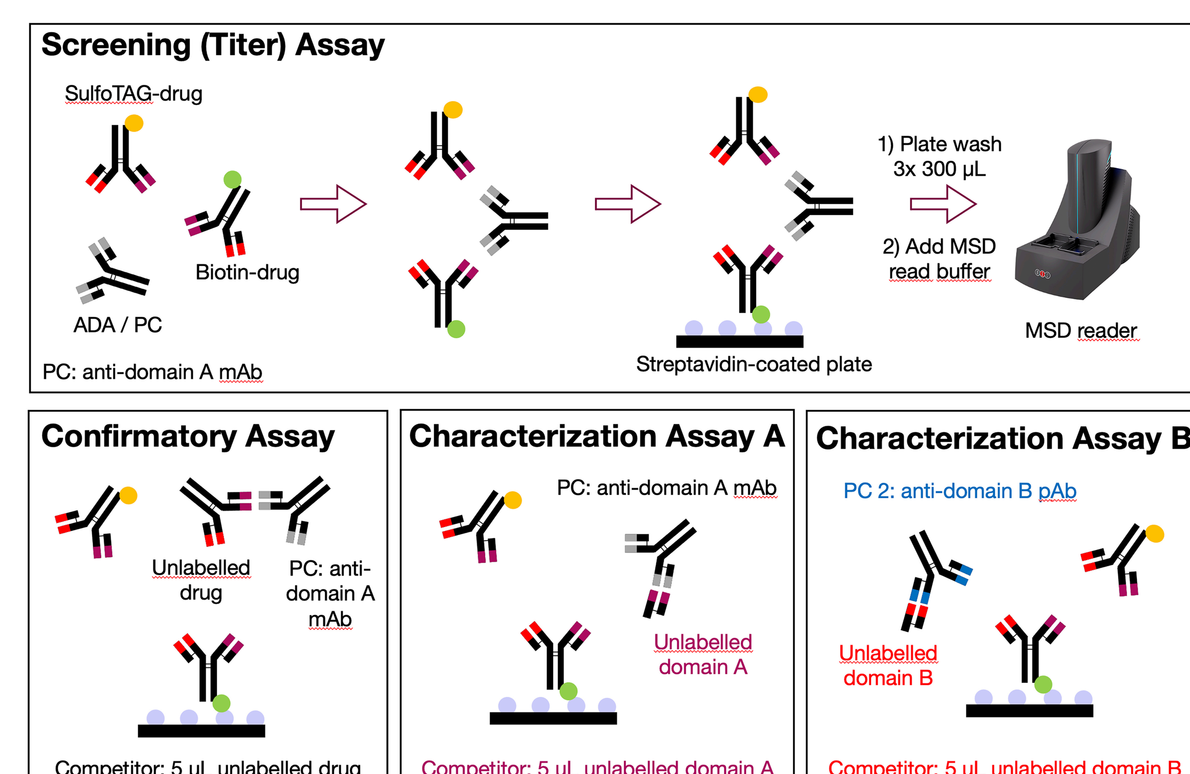


Figure 1. Schematic illustration of the method procedure in the different assay tiers.

Assay optimization was performed in singlicate using human serum as matrix. The following parameters were selected based on assay performance: 0.25 µg/mL Biotin-drug and 0.25 µg/mL SulfoTAG-drug, PBS 1% casein as assay buffer/blocking reagent and minimal required dilution (MRD) of 80 to reduce false-positive Target B interference (Figure 2). Low circulating drug level was not expected to interfere with the assay.

Target A

PC (ng/mL)	Target A (pg/mL)	RLU MRD 40	RLU MRD 80	S/N MRD 40	S/N MRD 80
0	0	89	81	1.00	1.00
0	1	90	75	1.01	0.93
0	1000	85	81	0.96	1.00
100	0	3366	1736	37.82	21.43
100	1	2981	1481	33.49	18.28
100	1000	3167	1531	35.58	18.90

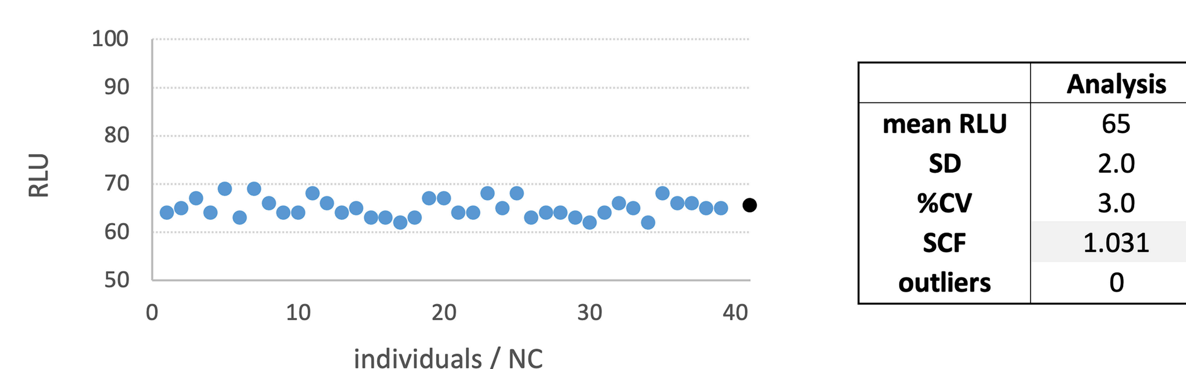
Target B

PC 2 (ng/mL)	Target B (ng/mL)	RLU MRD 40	RLU MRD 80	S/N MRD 40	S/N MRD 80
0	0	76	82	1.00	1.00
0	10	82	85	1.08	1.04
0	100	151	119	1.99	1.45
100	0	982	560	12.92	6.83
100	10	1068	554	14.05	6.76
100	100	939	543	12.36	6.62

Figure 2. Evaluation of target interference at different MRDs. PC: anti-domain A mAb; PC 2: anti-domain B pAb. Target B interference is marked in green.

BIOANALYTICAL QUESTION

The initial assay exhibited exceptional performance, with high sensitivity at MRD 80 and minimal matrix interference, which resulted in a preliminary cut point of < 1.05 SCF (Figure 3). The background noise of the assay was remarkably low, raising concerns about the assay's ability to detect biological variability as sample response of drug naïve samples approached the lower detection limit of the instrument. Specific assay parameters were investigated to assess their impact on inter-individual variability and assay background level.



	Analysis
mean RLU	65
SD	2.0
%CV	3.0
SCF	1.031
outliers	0

Figure 3. Preliminary cut point (CP) evaluation at MRD 80. 39 healthy individuals (blue); mean NC (black).

ASSAY OPTIMIZATION

Optimized Labelled Drug Concentrations

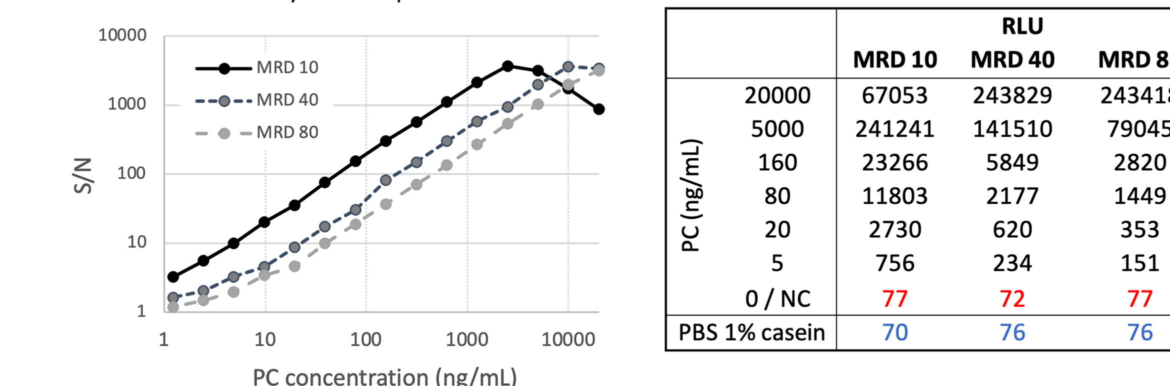
Different concentrations of capture and detection reagents (Figure 4) as well as three different labeling batches (data not shown) were evaluated for assay performance in diluent (PBS 1% casein) and matrix (human serum pool) at MRD 10. Remarkably, assay background was comparable in all tested conditions. The initial selected conditions, consisting of a concentration of 0.25 µg/mL for each conjugate, were maintained for subsequent analysis.

Conjugate amount		Total 1 µg/mL			Total 0.5 µg/mL			Total 0.25 µg/mL			Total 0.125 µg/mL		
B/S ratio		1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1
RLU													
PBS 1% casein	NC	74	73	72	70	76	74	71	72	70	73	69	71
	100 ng/mL PC	11232	10939	9642	11746	10686	9251	10783	9652	8837	8220	7625	7447
	500 ng/mL PC	56603	53943	47040	54897	51172	45478	44704	40304	37170	20634	20041	19299
matrix	NC	73	74	71	70	74	73	70	69	64	69	71	72
	100 ng/mL PC	12624	10228	10775	10955	10905	8944	11725	9800	8986	6406	6100	7085
	500 ng/mL PC	50662	49080	49923	45878	49988	39416	41585	34610	35080	17834	21496	16560

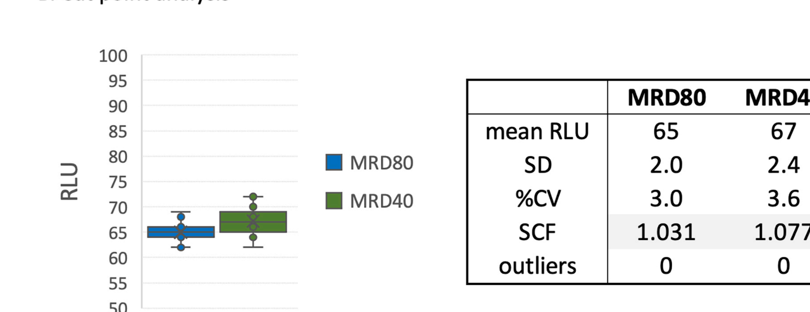
Figure 4. Impact of capture/detection reagent concentration on assay background.

Reduced MRD

Selection of the most optimal MRD is crucial in the development of ADA assay, as different sample dilution factors influence non-specific and/or specific binding of matrix components, thereby affecting sensitivity, drug/target interference, selectivity and inter-individual variability. Here decreased sample dilution marginally increased assay background and screening correction factor (SCF) (Figure 5). MRD80 was chosen for further analysis due to its effectiveness in reducing interference with Target B, a benefit that surpassed the minor increase in individual variability.



PC (ng/mL)	RLU MRD 10	RLU MRD 40	RLU MRD 80
20000	67053	243829	243418
5000	241241	141510	79045
160	23266	5849	2820
80	11803	2177	1449
20	2730	620	353
5	756	234	151
0 / NC	77	72	77
PBS 1% casein	70	76	76

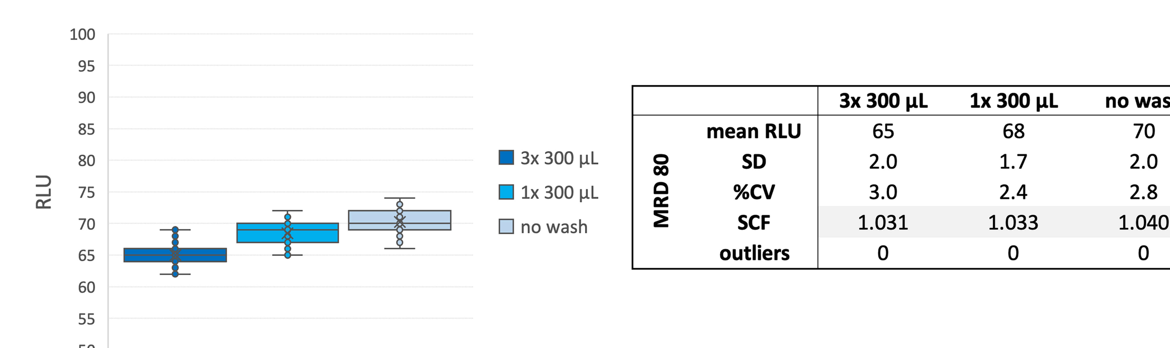


	MRD80	MRD40
mean RLU	65	67
SD	2.0	2.4
%CV	3.0	3.6
SCF	1.031	1.077
outliers	0	0

Figure 5. Impact of MRD on assay background, sensitivity and cut point.

Reduced Washes

The number of wash steps and cycles should be carefully controlled to prevent the washout of specific binding molecules with lower affinities and enable detection of biological variability. Here the number of wash cycles showed no impact on individual variability (Figure 6).



MRD 80	3x 300 µL	1x 300 µL	no wash
mean RLU	65	68	70
SD	2.0	1.7	2.0
%CV	3.0	2.4	2.8
SCF	1.031	1.033	1.040
outliers	0	0	0

Figure 6. Impact of wash cycles on individual variability.

Optimized Blocking/Assay Buffer

Another critical element is the selection of the proper assay buffer and blocking reagents used to prevent non-specific binding and reduce known interference. Three different buffers were compared for improved assay performance (Figure 7). While Buffer #3 only marginally increased assay background, it remarkably reduces positive target B interference, without compromising sensitivity. Sample acidification was also explored to enhance tolerance towards Target B, yet it resulted in a notable loss of sensitivity (Figure 7). Buffer #3 was thus selected for further analysis.

		RLU				S/N			
		no acidification			+ acid.	no acidification			+ acid.
PC (ng/mL)	Target B (ng/mL)	Buffer 1	Buffer 2	Buffer 3	Buffer 1	Buffer 1	Buffer 2	Buffer 3	Buffer 1
0	0	67	68	73	70	1.00	1.00	1.00	1.00
0	100	100	93	77	85	1.49	1.37	1.05	1.21
100	0	1428	1286	816	76	21.31	18.91	11.18	1.09
100	100	1512	1317	761	113	22.57	19.37	10.42	1.61

Figure 7. Impact of assay buffers and sample acidification on assay background, Target B interference (marked in green) and sensitivity.

Assay Performance in Patient Population

Selectivity was assessed using ten healthy and diseased individual matrices analyzed unspiked and spiked at pLPC (10 ng/mL). Assay performance was very comparable in both populations (Figure 8).

	Healthy individuals				Diseased individuals			
	RLU		S/N		RLU		S/N	
	blank	pLPC	blank	pLPC	blank	pLPC	blank	pLPC
pool	75	128	1.000	1.718	N/AP	N/AP	N/AP	N/AP
ind 1	74	121	0.993	1.624	77	123	1.034	1.651
ind 2	78	125	1.047	1.678	81	120	1.087	1.611
ind 3	76	125	1.020	1.678	80	129	1.074	1.732
ind 4	80	126	1.074	1.691	79	123	1.060	1.651
ind 5	83	122	1.114	1.638	78	128	1.047	1.718
ind 6	80	128	1.074	1.718	81	118	1.087	1.584
ind 7	80	114	1.074	1.530	78	125	1.047	1.678
ind 8	80	119	1.074	1.597	77	125	1.034	1.678
ind 9	81	136	1.087	1.826	78	125	1.047	1.678
ind 10	77	128	1.034	1.718	79	125	1.060	1.678
mean ind.	**79**	**124**	**1.059**	**1.670**	**79**	**124**	**1.058**	**1.666**
%CV ind.	**3.4%**	**4.8%**			**1.9%**	**2.7%**		

Figure 8. Evaluation of selectivity in healthy and diseased individual matrices.

Evaluation of Plate Homogeneity and Number of NCs for Singlicate Analysis

In Figure 8, we observed a minimal response discrepancy between individual matrices and the pool prepared from representative individuals (mean of 2 NC sets), resulting in a high false-positive rate (FPR) of drug-naïve samples due to the low SCP. We therefore evaluated whether NC set number and plate location influenced mean NC response and FPR by evaluating plate homogeneity at NC level. Additionally, fixation with 0.2% Glutaraldehyde solution as well as different plate lots were assessed.

In general, no plate drift was observed and NC responses throughout the plate were found to be homogenous (< 5% CV) in all tested conditions (data not shown), thus confirming the suitability of different plate lots and the unnecessity to include a fixation step. Notably, FPR was reduced when 4 NC singlicate sets were included and distributed throughout the plate. We therefore propose to include 2 NC sets at the beginning and end of the plate together with other PC levels, and 2 NC sets in the middle of the plate.

CONCLUSIONS & DISCUSSION

Throughout method development, we confirmed the robustness and suitability of the assay. As a result, we confidently confirmed the assay parameters despite the low established cut points (1.0404 SCF, 9.0% confirmatory CP, 12.1% CP characterization A, 4.0 % iCP characterization B). Sensitivities were considerably below the requested 100 ng/mL[1] (1-10 ng/mL) in all assay tiers and for both positive controls.

Remarkably, SCF determined in development (1.0404) was lower than the inter-assay precision of NC (4.3% CV). This observation relates to ongoing discussion[2] surrounding the clinical relevance of statistically determined CPs that fall below the assay variability of the NC.

REFERENCES

[1] FDA, Immunogenicity Testing of Therapeutic Protein Products—Developing and Validating Assays for Anti-Drug Antibody Detection, 2019

[2] Examples: Bioanalysis (2018) 10(24), 1973–2001