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Using Anti-Drug-Antibody Screening Assay to Resolve Selectivity Issues in Toxicokinetic Ligand-Binding Assay

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INTRODUCTION

Selectivity evaluation for regulatory toxicokinetic (TK) quantitative ligand-binding assays (LBAs) is required to meet accuracy requirements for at least 80% of the individual selectivity matrix lots. Due to the complexity of biological matrices, this parameter often requires extensive method optimization to achieve the required level of accuracy. Individual selectivity lots, spiked with the drug at the level of high-quality control (HQC) and lower limit of quantitation (LLOQ) concentrations, are back-calculated from a regressed calibration curve that has been prepared from a pooled matrix of multiple individual lots. If the pooled matrix contains individual lots with interfering factors, such as pre-existing/cross-reacting antibodies or soluble drug targets, then the back-calculated concentration of selectivity samples might be inaccurately represented. Therefore, a routine screening of individual matrix lots in an anti-drug antibody (ADA) assay can be used to assess the presence of such interfering pre-existing/cross-reacting antibodies, and those lots can be excluded from the TK pooled matrix.

METHODS

ADA and TK methods were developed to support a TK study for a large molecule biologic (drug) in cynomolgus monkey serum.

TK Method: A colorimetric quantitative sandwich ELISA method was developed. The ELISA consisted of a plate coated with capture reagent (drug target) and an HRP anti-human IgG, monkey IgG adsorbed detection antibody. The TK ELISA method was developed to be stepwise: the first step included incubation of the drug spiked serum at minimum required dilution (MRD) with the capture reagent followed by incubation of the captured drug with the detection antibody solution. Signal was developed with TMB and stopped with acidic stop solution. The developed plate was read on BioTek Synergy plate reader at 450 nm wavelength and the drug concentration was calculated from a 4-parameter logistic calibration curve regression with 1/y2 weighting.

ADA Method: A bridging electrochemiluminescence immunoassay (ECLIA) method with acid dissociation was developed. The biotinylated drug was used to capture the ADA and the ruthenylated drug was used to detect the ADA. A polyclonal antibody raised against the drug and spiked into treatment-naive pooled monkey serum was used as a positive control (PC), while the unspiked treatment-naive pooled monkey serum was used as a negative control (NC). The ADA ECLIA method consisted of two steps: the first step included treatment of the PC and NC samples with a weak acid, and then the acid-treated samples were neutralized with a basic master mix solution containing the capture and detection reagents. The biotinylated drug-ADA-ruthenylated drug complexes were captured on a streptavidin Meso Scale Discovery (MSD) plate and the MSD platform was used to detect the ECL signal after an electrochemical reaction of MSD Read Buffer T (1X) and ruthenium.

INITIAL TK SELECTIVITY RESULT

Pooled cynomolgus monkey serum, consisting of 36 randomly selected serum lots from treatment-naive animals (male and female), was initially used in preparation of a TK calibration curve and quality controls (QCs). Individual selectivity samples spiked at the LLOQ level (highlighted) over-recovered when back-calculated against this calibration curve and therefore did not meet acceptable accuracy criteria.

Sample ID	Nominal Conc. (ng/mL)	450nm (Mean)	CV (%)	Calculated Conc. (ng/mL)	%RE
Quality Controls					
ULQC	10000	3.946	0.3	9640	-3.6
HQC	7692	3.879	0.4	8110	5.4
MQC	5000	3.716	0.6	5880	17.7
LQC	100	0.232	0.5	109	9.2
LLQC	39	0.125	4.1	40.8	4.6
Calibration Standards and Matrix Blank					
STD1 (ULOQ)	10000	3.925	0.1	9100	-9
STD2	8000	3.857	0.1	7710	-3.7
STD3	4000	3.498	1.4	4300	7.5
STD4	2000	2.733	2.7	2060	3.1
STD5	1250	2.024	0.6	1210	-3.1
STD6	625	1.196	0.2	622	-0.4
STD7	313	0.614	2.2	310	-0.8
STD8	156	0.326	1.8	162	3.7
STD9	78	0.183	3.7	80.2	2.8
STD10 (LLOQ)	39	0.125	5	40.8	4.7
STD11	20	0.094	0.2	15	-25.1
Blank	0	0.067	11.6	BLQ	NA

Table 1. Standards and QCs prepared in pooled serum of randomly selected lots

Selectivity ID	Nominal Conc. (ng/mL)	450nm (Mean)	CV (%)	Calculated Conc. (ng/mL)	%RE
Sel-1 HQC	7692	3.816	2.0	7161	-6.9
Sel-1 LLOQ	39	0.161	5.4	66	69.2
Sel-2 HQC	7692	3.822	0.1	7149	-7.1
Sel-2 LLOQ	39	0.171	1.9	72.8	86.7
Sel-3 HQC	7692	3.852	0.2	7623	-0.9
Sel-3 LLOQ	39	0.172	4.1	73.1	87.4
Sel-4 HQC	7692	3.86	0.2	7768	1.0
Sel-4 LLOQ	39	0.172	4.1	73.3	87.9
Sel-5 HQC	7692	3.842	0.8	7472	-2.9
Sel-5 LLOQ	39	0.172	4.7	73.4	88.2

Table 2. Selectivity samples back-calculated against calibration curve in Table 1

Attempts to resolve the selectivity failure at the LLOQ level included: testing of different blocking and assay buffers, raising the LLOQ level, and reducing the incubation times at binding steps. Although one buffer improved the accuracy at the LLOQ level for the selectivity samples, it was not enough to meet the accuracy acceptance criteria for the selectivity lots.

ADA BIOLOGICAL OUTLIERS

Twenty individual lots of cynomolgus monkey serum were screened from treatment-naive animals and two biological outliers (highlighted) were detected. The rest of the individual serum lots showed a low ECL signal of similar magnitude.

Sample ID	ECL Intensity	
	Run MD7	Run MD8
NC (Set1)	49	62
NC (Set2)	48	57
Mean	49	60
AB7658	93	116
AB7653	45	61
AB7654	43	57
AB7659	43	56
AB7604	42	53
AB7616	52	63
AB7610	39	NA
AB7611	43	53
AB7610	40	NA
AB7609	46	54
AB7635	43	54
AB7639	42	54
AB7647	47	59
AB7620	52	65
AB7613	43	59
AB7619	59	NA
AB7605	45	55
AB7617	172	194
AB7644	NA	53
AB7642	NA	57
AB7650	NA	56
25th percentile	43	54
75th percentile	51	61
Upper side of cut off value for an outlier	63	72
Lower side of cut off value for an outlier	31	44
Outlier for lower side	No	No
Outlier for upper side	Yes	Yes

NC: Negative control
ECL: Electrochemiluminescence

Table 3. ADA screening results of individual serum lots

These two biological outliers from treatment-naive animals suggested that pre-existing/cross-reacting ADAs or soluble drug targets can be encountered in some animals. This led to the hypothesis that the pooled serum used to prepare the calibration curve and QCs might include some ADAs or soluble drug targets. Having these lots within the matrix pool preparation may have added interference factors in the TK assay, which led to an under-recovery of the calibration curve. This would explain the over-recovery of the individual selectivity LLOQ against this calibration curve.

The next step included a preparation of pooled serum without the ADA biological outliers. This ADA assay-selected serum was tested in a TK assay, which included a calibration curve and five levels of QCs prepared in the ADA assay-selected serum, two individual serum lots for selectivity assessment at three concentration levels, and lower quality control (LQC) and LLOQ prepared in the previous lot of pooled serum consisting of randomly selected individuals (highlighted in Table 5).

TK POOLED MATRIX WITH ADA SCREENED LOTS

Sample ID	Nominal Conc. (ng/mL)	Pooled Matrix Type	450nm (Mean)	CV (%)	Calculated Conc. (ng/mL)	%RE
Quality Controls						
ULQC	10000	Selected in ADA Assay	3.576	1.8	9085	-9.1
HQC	7692	Selected in ADA Assay	3.393	0.2	7612	-1.0
MQC	5000	Selected in ADA Assay	2.979	0.0	5292	5.8
LQC	100	Selected in ADA Assay	0.168	1.2	96.6	-3.4
LLQC	39	Selected in ADA Assay	0.091	3.0	37.2	-4.6
Calibration Standards and Matrix Blank						
STD1 (ULOQ)	10000	Selected in ADA Assay	3.693	2.0	10252	2.5
STD2	8000	Selected in ADA Assay	3.398	0.0	7649	-4.4
STD3	4000	Selected in ADA Assay	2.685	0.3	4156	3.9
STD4	2000	Selected in ADA Assay	1.787	1.3	1977	-1.2
STD5	1250	Selected in ADA Assay	1.303	2.7	1243	-0.6
STD6	625	Selected in ADA Assay	0.762	3.0	621	-0.6
STD7	313	Selected in ADA Assay	0.428	1.7	310	-1.0
STD8	156	Selected in ADA Assay	0.253	1.7	164	4.9
STD9	78	Selected in ADA Assay	0.144	1.3	77.7	-0.4
STD10 (LLOQ)	39	Selected in ADA Assay	0.092	1.5	38.4	-1.4
STD11	20	Selected in ADA Assay	0.067	0.1	19.5	-2.7
Blank	0	Selected in ADA Assay	0.045	9.8	BLQ	NA

Table 4. Standards and QCs prepared in pooled serum of ADA assay selected lots

Sample ID	Nominal Conc. (ng/mL)	Pooled Matrix Type	450nm (Mean)	CV (%)	Calculated Conc. (ng/mL)	%RE
LQC	100	Randomly Selected	0.084	0.4	32.2	-67.8
LLQC	39	Randomly Selected	0.054	0.0	9.33	-76.1
Sel-1	39	Individual lot 1	0.096	2.6	41.3	5.9
Sel-1	100	Individual lot 1	0.169	2.2	97.5	-2.5
Sel-2	100	Individual lot 2	0.168	2.3	96.7	-3.3
Sel-1	200	Individual lot 1	0.3	2.4	202	1.2
Sel-2	200	Individual lot 2	0.298	3.8	201	0.4

Table 5. Selectivity samples back-calculated against calibration curve in Table 4

The LQC and LLOQ preparation in pooled serum consisting of randomly selected individuals demonstrated under-recovery when back-calculated against the calibration curve prepared in the ADA assay-selected serum. The under-recovery supported the hypothesis that the randomly selected individuals used to make the pooled serum included some individuals with interfering factors. The ADA assay demonstrated that these individual serums with interfering factors make up only 10% of the population, and are therefore not representative of the majority. Excluding these outliers allowed for selectivity QCs to recover appropriately without any further assay optimization.

FINAL TK SELECTIVITY RESULT

Selectivity was repeated with randomly selected and untested individual serum lots. They were then back-calculated against a calibration curve prepared in the pooled serum of individual lots that was selected in the ADA assay. One hundred percent of the selectivity lots met acceptance criteria at all QC levels.

Nominal Concentrations (ng/mL)		Sel-LLOQ			Sel-HQC		
		39			7,692		
Selectivity ID	Run ID	Conc. (ng/mL)	*%CV	*%RE	Conc. (ng/mL)	*%CV	*%RE
Sel-6	MD13	34.3	1.4	-12.0	7850	0.4	2.1
Sel-7		35.3	0.8	-9.5	7740	2.1	0.6
Sel-8		45.2	7.7	15.8	8350	0.4	8.5
Sel-9		35.2	3.0	-9.8	7480	1.4	-2.7
Sel-10		36.1	2.1	-7.5	7840	1.5	1.9
Sel-11		34.6	0.5	-11.4	7870	0.8	2.4
N		6			6		
Mean		36.8			7860		
SD		4.17			283		
%CV		11.3			3.6		
%RE		-5.6			2.2		

Table 5. Randomly selected and untested individual selectivity samples back-calculated against calibration curve prepared in pooled serum of ADA assay selected lots

CONCLUSIONS

Although the presence of pre-existing/cross-reacting ADA is well-known, the ADA assay is not routinely used to support the development of the TK assay. These pre-existing/cross-reacting ADAs or soluble drug targets can cause selectivity failure via specific interference when used in a pooled serum to prepare the TK calibration curve and QCs, as demonstrated above. Relying solely on the TK assay method, development data are not always sufficient to resolve assay issues. Results obtained from the ADA assay can be useful tools to better understand the interference observed in the TK assay. Using the screening data from the ADA assay allowed for identification of treatment-naive individuals with pre-existing/cross-reactive ADAs or soluble drug targets, which after exclusion from the pooled TK serum resolved the selectivity failure at LLOQ. In conclusion, screening for ADA biological outliers among individual matrix lots may be an effective tool for evaluating the suitability of individual lots selected for the preparation of pooled serum in TK assays.