An Evaluation of the Effect of Duplicate- and Single- Well Analyses on Assay Cut Point Calculations and Characterization Experiments in Preclinical Immunogenicity Assays

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PURPOSE

Evaluate the feasibility of single-well analysis over duplicate-well analysis in electrochemiluminescent immunoassays in less variable populations, such as preclinical populations.

BACKGROUND

Historically, immunoassay technology, reagents and labeling techniques had systemic variability which required duplicate-well analysis to ensure accurate and precise analytical results. This led to duplicate-well analysis becoming a standard practice in pharmacokinetic, quantitative biomarker and immunogenicity immunoassays. Duplicate-well analyses are used in immunogenicity immunoassays to construct statistical distributions to calculate assay cut points (CP) as well as for overall acceptance and rejection of plates.

However, duplicate-well analysis has increasingly become unnecessary due to the advances in immunoassay technologies and critical reagent generation. These advances have led to improved assay precision and robustness and opened the possibility of single-well analysis without sacrificing data quality and integrity.

OBJECTIVES

- Statistically demonstrate that the data distributions of each analytical duplicate and the duplicate means are equivalent.
- Demonstrate that assay cut points generated from each analytical duplicate and the duplicate means are functionally equivalent.
- Demonstrate that the experimental conclusions when using each analytical duplicate and the duplicate means are equivalent

METHODS

Assay validation data from a rat preclinical immunogenicity assay were used as a case study dataset. Validation experiments included were screening (Tier 1) and confirmatory (Tier 2) tier cut point generation, drug tolerance, matrix selectivity, analytical sensitivity, titration precision, freeze/thaw and thawed sample stability and prozone evaluation.

All experimental results included screening (signal to noise ratio) and confirmatory (%inhibition between samples without drug and in the presence of excess drug) values. All experiments were performed in duplicate wells which produced identical datasets for analytical duplicate 1 (D1), duplicate 2 (D2) and mean duplicate value (MDV).

The means of the percent inhibition (% Inhib) and signal to noise (S/N) ratio distributions for D1, D2, and MDV were compared using ordinary least squares (OLS) regression. To identify insignificant differences, we used $\alpha = 0.10$ as the threshold for statistical significance. Quantile regression was then used to compare the 25th, 50th, and 75th percentiles of the D1, D2, and MDV distributions.

Screening and confirmatory cut point (CP) analyses were performed on the D1, D2, and MDV data sets and were evaluated independently using the corresponding CP value for Tier 1 and Tier 2.

To demonstrate functional D1, D2, and MDV data set comparability, positive control classification and assay characterization experimental results, e.g., drug and target tolerance, matrix interference, selectivity, sensitivity and stability, were examined to identify any differences in experimental conclusions.

RESULTS

No significant differences were observed in the mean of the distributions of D1, D2, and MDV datasets. The OLS regression *p*-value results ranged from 0.914 - 0.996 in rat which demonstrates that the means of the datasets are not significantly different. The quantile regression comparing the 25th, 50th, and 75th percentile distributions of the D1, D2, and MDV datasets yielded *p*-values ranging from 0.262 - 0.944 in rat, which further demonstrates that the datasets are not statistically significantly different.

CP analyses in the D1, D2, and MDV datasets produced Tier 1 CP values of 1.16, 1.19 and 1.19 (S/N), and Tier 2 CP values of 25.0%, 29.1%, and 27.3% inhibition, respectively. The Tier 1 and Tier 2 CP values were not statistically significant with *p*-values from 0.414 - 1.00.

Examination of positive control classification and assay characterization experimental results in the D1, D2, and MDV datasets determined that there were no differences in experimental conclusions.

CONCLUSION

No statistical or functional differences in experimental results were observed between the D1, D2, and MDV datasets. These results support the use of single well analysis in electrochemiluminescent immunoassays which would increase laboratory throughput and potentially reduce required sample volumes without impacting data accuracy and quality.

REFERENCES

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Singlet analysis yields equivalent results as duplicate analysis in preclinical immunogenicity assessment.

	Signal to Noise		Percent Inhibition	
	Comparing Differences at the Mean [coefficient, (standard error)]	Comparing Differences at the Quartiles [coefficient, (standard error)]	Comparing Differences at the Mean [coefficient, (standard error)]	Comparing Differences at the Quartiles [coefficient, (standard error)]
Mean vs. Duplicate 1	-0.004 (0.832)	Q25: 0.001 (0.948)	0.135 (0.832)	Q25: 1.33 (0.948)
		Q50: -0.012 (0.248)		Q50: -1.19 (0.352)
		Q75: -0.005 (0.819)		Q75: -0.005 (0.715)
Mean vs. Duplicate 2	0.003 (0.887)	Q25: 0.008 (0.508)	0.142 (0.887)	Q25: 1.23 (0.508)
		Q50: -0.006 (0.458)		Q50: -0.00564 (0.701)
		Q75: -0.012 (0.202)		Q75: -0.0121 (0.202)
Duplicate 1 vs. Duplicate 2	0.006 (0.728)	Q25: 0.007 (0.532)	0.007 (0.996)	Q25: -0.099 (0.927)
		Q50: 0.006 (0.687)		Q50: 0.350 (0.819)
		Q75: -0.007 (0.689)		Q75: 0.108 (0.944)

Table 1: Ordinary Least Squares and Quantile Regression results showing no significant difference in rat data using duplicate 1, duplicate 2, and duplicate mean observations. Q25 = 25th Percentile, Q50 = 50th Percentile, Q75 = 75th Percentile.

Ordinary Least Squares: $y_i = \beta_0 + \beta_1 x_{i1} + \beta_p x_{ip}$
Quantile Regression: $Q(yi) = \beta_0(\tau) + \beta_1(\tau)xi_{1} + \beta_p(\tau)xip$
where i = 1, ..., n.

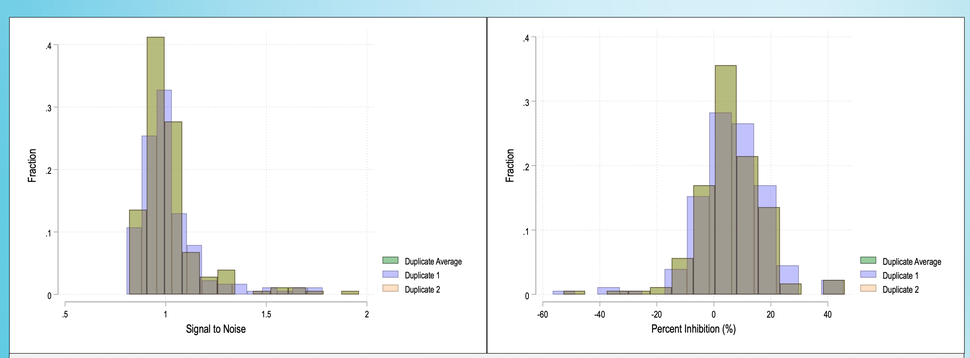

Figure 1: Distributions of signal to noise ratios and percent inhibition of duplicate 1, duplicate 2, and the duplicate mean show substantial overlap.

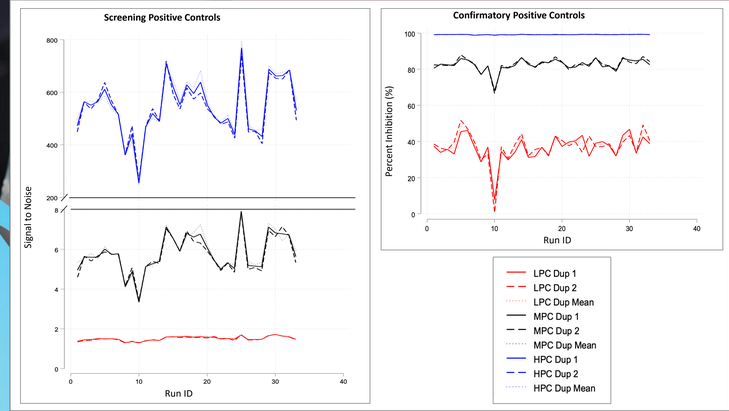

Figure 2: Percent inhibition from screening and confirmatory runs showed similar performance of HPC, MPC, and LPC for both duplicates and the duplicate mean on all runs.
NOTE: Due to high level of response to the dosage with high positive controls, we had to adjust and manipulate the scale for signal to noise.

Experiment	Tier	Duplicate 1	Duplicate 2	Mean Duplicate Value
Cut Point Determination*	1	1.19 SNR	1.19 SNR	1.16 SNR
	2	29.05 %Inhib	27.29 %Inhib	25.02 %Inhib
Sensitivity (ng/mL)	1	3.73	3.64	3.68
	2	6.36	6.34	6.37
Selectivity (n=10 each level)	1	All acceptable in blank, LPC and HPC spiked samples (n=10 each level)		
	2			
Intra-Assay Precision (Tier 1 - SNR %CV; Tier 2 - %Inhib %CV)	1	< 9.6	< 12.1	< 10.4
	2	< 17.4	< 18.5	< 10.5
Drug Tolerance: 100 ng PC/mL	1	> 20 ug Drug/mL	> 20 ug Drug/mL	> 20 ug Drug/mL
	2	> 10 ug Drug/mL	> 10 ug Drug/mL	> 10 ug Drug/mL
Target Tolerance: Blank samples	1	< 10 ng Target/mL	< 10 ng Target/mL	< 10 ng Target/mL
	2	10 ng Target/mL	10 ng Target/mL	10 ng Target/mL
Room Temperature Stability	1	All levels tested were stable for 24 hours at Room Temperature		
	2			
Freeze/Thaw Stability	1	All levels tested were stable through 6 Freeze/Thaw Cycles		
	2			
Prozone/Hook Effect	1	No Hook Observed		
	2			

Table 2: Validation data for ADA assay in rat serum. Singlet, duplicate, and mean values showed no statistical differences.
Tier 1: Screening Tier
Tier 2: Confirmatory Tier
* A Tukey Coefficient of 3 was used for outlier removal.

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