Partial Method Validation of Biomarker Assays to Determine Adrenocorticotropic Hormone (ACTH) Concentrations in Rat and Canine Serum in Support of Pre-Clinical Endocrinology Drug Development

Michael Sonnenfelt[1], Taylor Blizzard[1], Kim Annis[1], Barry Jones[2], Rosa Luo[2], Susan Zondlo[1], Jian Wang[2]
[1]QPS, Newark, DE; [2]Crinetics Pharmaceuticals, San Diego, CA

NOVEL ASPECT

Although endocrine diseases such as Cushing's disease or ectopic adrenocorticotropic hormone (ACTH) syndrome are relatively rare in the human population, there still exists a need to understand how to treat these ailments and even potentially reverse the acute symptoms caused by the disease. In order to understand how exploratory endocrinology drugs affect endogenous biomarkers that contribute to symptom severity, a reliable and reproducible method of measuring these biomarkers must be identified. Additionally, to ensure that these exploratory drugs are not toxic in the human population, animal models (such as the Sprague Dawley rat and canine) can help inform the potential toxicity of a drug during pre-clinical trials. Methods using a Luminex 200 were partially validated at QPS to determine the ACTH concentration in Sprague Dawley rat serum and canine serum optimizing commercially-available research use only (RUO) assay kits.

RAT & CANINE METHOD SUMMARY

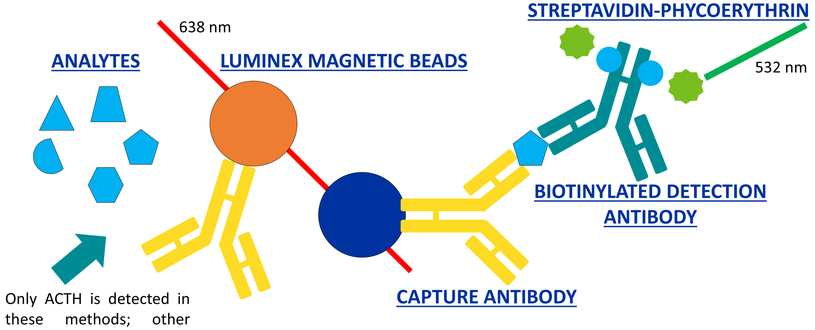
Method Summary

In these assays, ACTH is immobilized by antibodies coated on color-coded magnetic beads. Due to the endogenous nature of ACTH, the standard curves are prepared in the surrogate matrix (assay buffer) using recombinant ACTH. To assess the true reproducibility of the method, quality control (QC) samples prepared by spiking recombinant ACTH into surrogate matrix (buffer QCs) as well as quality control samples made from rat or canine serum containing endogenous ACTH (endogenous QCs) were prepared. The recombinant or endogenous ACTH present in the standards, buffer QCs and endogenous QCs binds to biotinylated antibodies on magnetic beads. Next, the immobilized ACTH is detected using a streptavidin-phycoerythrin conjugate. The beads are read by the Luminex 200 to determine a fluorescent intensity that is proportional to the ACTH concentration. The method was developed to precisely and accurately measure ACTH in pre-dose and post-dose samples from Sprague Dawley rats and canines collected during a variety of pre-clinical studies developing exploratory endocrinology drugs. Partial validation tests were performed on normal healthy serum from both Sprague Dawley rats and canines, and included precision and accuracy testing, matrix selectivity, parallelism, room temperature stability, and freeze thaw stability.

Note: In these methods, only beads specific for ACTH were used. Beads for other pituitary/stress hormones are available for these plexes but were excluded in this analysis.

Bioanalytical Method		Determination of ACTH in Rat Serum	Determination of ACTH in Canine Serum
Materials used for Standards	Standard Material	Rat Stress Hormone Standard	Canine Pituitary Expanded Panel Standard
	Surrogate Matrix	Assay Buffer	Assay Buffer
Qualified Assay Range	Range	1.37 pg/mL to 1000 pg/mL	3.43 pg/mL to 2500 pg/mL
	LLOQ	1.37 pg/mL	3.43 pg/mL
	ULOQ	1000 pg/mL	2500 pg/mL
Material used for endogenous quality controls (eQCs) and concentration	Matrix	Sprague Dawley rat serum (for LQC, MQC, and HQC)	Matrix: Canine serum (for EQC1, EQC2, and Spiked EQC3
	LQC	LQC1: 29.6 pg/mL (Lot# RAT516315) LQC2: 26.0 pg/mL (Lot# RAT516330)	EQC1: 9.23 pg/mL (Lot#BGL143773)
	MQC	MQC1: 109 pg/mL (Lot# RAT516349) MQC2: 93.8 pg/mL(Lot# RAT316299)	EQC2: 5.87 pg/mL (Lot#BGL143754)
	HQC	HQC1: 312 pg/mL (Lot# RAT316311) HQC2: 164 pg/mL (Lot# RAT316324)	Spiked EQC3: 312 pg/mL (Lot#TCB230321-01)
Material used for buffer quality controls (bQCs) and concentration	ACTH Stock	Rat Stress Hormone Standard & Kit Controls	Canine Pituitary Expanded Panel Standard & Kit Controls
	Surrogate Matrix	Assay Buffer	Assay Buffer
	LLOQ1 QC	1.37 pg/mL (Recombinant Spike)	10.3 pg/mL (Recombinant Spike)
	LLOQ2 QC	4.12 pg/mL(Recombinant Spike)	3.43 pg/mL (Recombinant Spike)
	BQC1	21.5 pg/mL (Kit Control 1)	47.5 pg/mL (Kit Control 1)
	BQC2	108 pg/mL (Kit Control 2)	388 pg/mL (Kit Control 2)
	BQC3	NA	1000 pg/mL(Recombinant Spike)
	ULOQ	1000 pg/mL (Recombinant Spike)	2500 pg/mL (Recombinant Spike)
MRD	MRD	4-fold	2-fold
	Sample Volume	20 µL	40 µL
Data Acquisition	Equipment	BioRad Luminex 200	
	Program	BioRad Bioplex Manager version 11.0	
	Program	Watson™ LIMS Version 7.4.1	
Data Analysis	Regression	4-parameter (Logistic Auto-estimate)	
	Weighting	1/Y	

LUMINEX MECHANISM OF ACTION



Only ACTH is detected in these methods; other analytes are not bound.

Biomarker assays benefit greatly from **consistency between RUO kits** and matrix having **measurable endogenous concentrations** of the biomarker of interest.

When faced with **kit lot-to-lot variability** and eQC material that **does not span the full length of the standard curve range**, additional considerations must be explored.

Source and lot of reagents			
	Kit Name	Milliplex Rat Stress Hormone Magnetic Bead Panel	Canine Pituitary Expanded Panel
	Catalog Number	RSHMAG-69K	CANPIT-96K
	Lot Number	3931433	3990441
	Expiration Date	30 Apr 2024	31 Jan 2024
	Storage Conditions	4°C	4°C

RAT & CANINE METHOD RESULTS

Method Qualification Parameters		Sprague Dawley Rat Method				Canine Method			
Qualification Test	Target Criteria	Result							
Standard calibration curve performance for all Qualification runs	Number of standards from LLOQ to ULOQ	7				7			
	Cumulative accuracy (%RE) from LLOQ to ULOQ	-11.9 to 16.1%				-10.6% to 13.1%			
	Cumulative precision (%CV) from LLOQ to ULOQ	≤ 5.0%				≤ 3.0%			
Performance of QCs during accuracy and precision runs	Precision (%CV) ≤ 20.0% for each QC; Accuracy (%RE) ≤ ± 25.0 (±25.0% for the LLOQ and ULOQ) of the nominal QC concentration; [a] %RE values are "0.0" as the Inter-batch runs are used to determine the nominal concentration of the matrix QCs.	QC	%CV	%RE	%TE	QC	%CV	%RE	%TE
		LLOQ1	6.5%	11.7%	18.2%	LLOQ1	2.9%	-3.0%	5.9%
		LLOQ2	6.3%	-10.7%	17.0%	LLOQ2	5.5%	20.7%	26.2%
		LQC1	8.2%	0.0%[a]	8.2%	EQC1	8.0%	0.0%[a]	8.0%
		LQC2	8.8%	0.0%[a]	8.8%				
		MQC1	3.1%	0.0%[a]	3.1%	EQC2	10.4%	0.0%[a]	10.4%
		MQC2	14.0%	0.0%[a]	14.0%				
		HQC1	4.2%	0.0%[a]	4.2%	Spiked EQC3	4.5%	0.0%[a]	4.5%
		HQC2	5.2%	0.0%[a]	5.2%				
		BQC1	5.3%	-0.5%	5.8%	BQC1	4.0%	0.2%	4.2%
		BQC2	2.6%	-1.9%	4.5%	BQC2	4.6%	2.1%	6.7%
		BQC3	NA	NA	NA	BQC3	2.1%	-3.3%	5.4%
		ULOQ	10.6%	-0.4%	11.0%	ULOQ	7.5%	10.4%	17.9%
Parallelism	The back calculated concentration of ACTH must be within ± 25.0% of the nominal concentration of the 4-fold diluted sample (rat) or 2-fold diluted sample (canine)	• Parallelism was observed at 4-fold, 8-fold, and 16-fold dilution factors in both lots tested. • Samples may be diluted 4, 8, or 16-fold.				• Parallelism was not observed in low-endogenous tested lots due to samples measuring BQL. • Two incurred samples with endogenous ACTH were tested. Both lots were parallel at the 2-fold and 4-fold dilution. • Samples may be diluted at 2-fold or 4-fold.			
Selectivity & matrix effect	3 normal serum lots for each matrix tested unspiked and spiked at the assay LLOQ and spiked near the mid range of the assay curve.	• % Recovery at the 1.37 pg/mL test was consistent between normal lots (mean of 87.8%). • % Recovery at the 145 pg/mL test was consistent between normal lots (mean of 74.6%).				• % Recovery at the 3.43 pg/mL test was consistent between normal lots (93.6%). • % Recovery at the 500 pg/mL test was inconsistent between normal lots (mean of 143.0%)			

DISCUSSION

- Two different assays were successfully developed at QPS to measure ACTH in either Sprague Dawley rat serum or canine serum. The partial validations that were performed show that these methods can measure pre-clinical samples both precisely and accurately.
- The partial validations also show that samples can undergo stressed stability conditions such as freeze thaw cycles or extended time at room temperature without affecting the reliability of the ACTH measurement.
  - Incurred sample analysis strongly informs that samples having measurable endogenous are required to obtain reliable qualification test results.
- Although the partial validation follows a methodology similar to that used in traditional pharmacokinetic assay validations, additional considerations must be made when measuring endogenous biomarkers with RUO assay kits.
  - The biomarker assays utilized for exploratory analysis are relative measurements noting the changes between pre-dose samples and post-dose samples since the recombinant protein (biomarker standard) and the endogenous protein may not be identical.
  - Additionally, changes in standard material from different production lots of RUO kits may affect the robustness of the assay.
  - The usefulness and limitations of using either buffer QCs or endogenous QCs and the strategy will also be discussed.
- Despite these challenges, exploratory biomarker assays can still provide useful information in determining the effectiveness of drug candidates.