

## NOVEL ASPECT

Although endocrine diseases such as Cushing's disease or ectopic adrenocorticotrophic 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

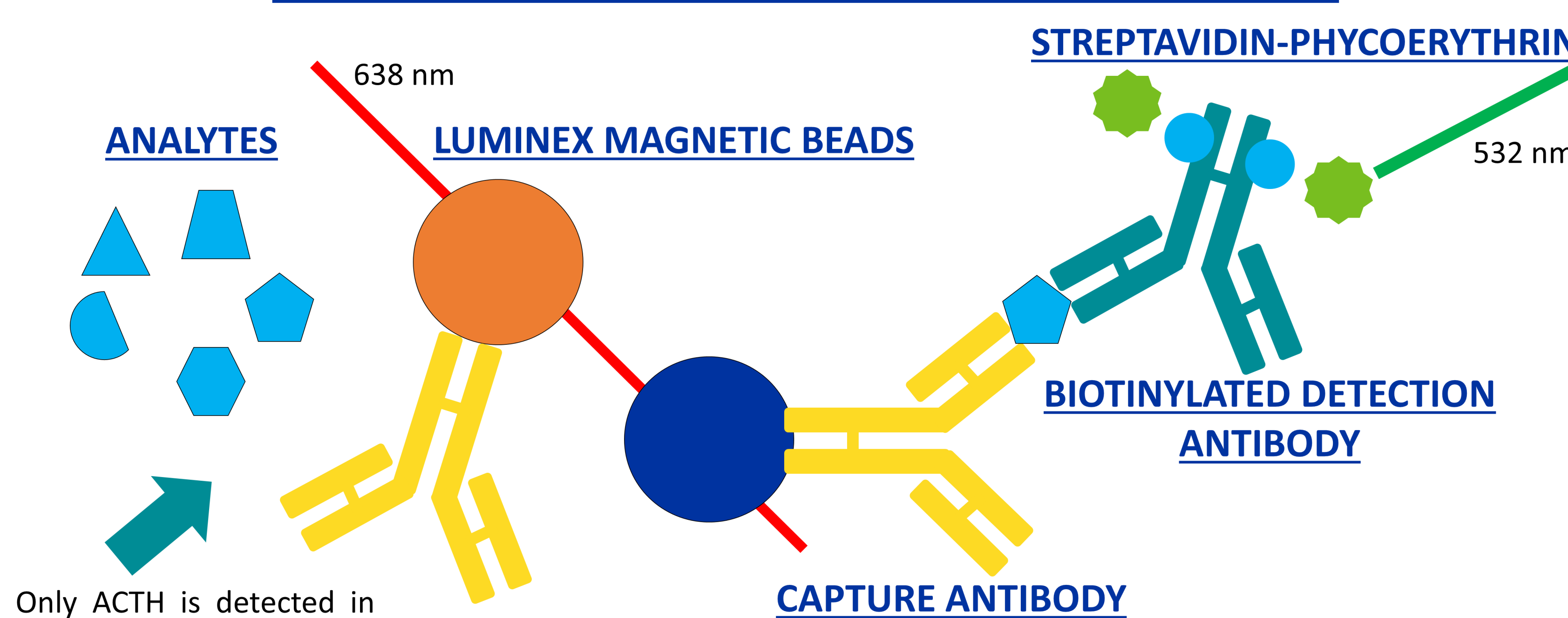
### Method Summary

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                        |
|---|--------------------------|--|--|
| <b>Materials used for Standards</b>   | <b>Standard Material</b> | Rat Stress Hormone Standard  | Canine Pituitary Expanded Panel Standard                     |
|   | <b>Surrogate Matrix</b>  | Assay Buffer   | Assay Buffer   |
| <b>Qualified Assay Range</b>  | <b>Range</b>             | 1.37 pg/mL to 1000 pg/mL   | 3.43 pg/mL to 2500 pg/mL                                     |
|   | <b>LLOQ</b>              | 1.37 pg/mL   | 3.43 pg/mL   |
|   | <b>ULOQ</b>              | 1000 pg/mL   | 2500 pg/mL   |
| <b>Material used for endogenous quality controls (eQCs) and concentration</b> | <b>Matrix</b>            | Sprague Dawley rat serum (for LQC, MQC, and HQC)                       | <b>Matrix</b> Canine serum (for EQC1, EQC2, and Spiked EQC3) |
|   | <b>LQC</b>               | LQC1: 29.6 pg/mL (Lot# RAT516315)<br>LQC2: 26.0 pg/mL (Lot# RAT516330) | <b>EQC1</b> 9.23 pg/mL (Lot#BGL143773)                       |
|   | <b>MQC</b>               | MQC1: 109 pg/mL (Lot# RAT516349)<br>MQC2: 93.8 pg/mL (Lot# RAT316299)  | <b>EQC2</b> 5.87 pg/mL (Lot#BGL143754)                       |
|   | <b>HQC</b>               | HQC1: 312 pg/mL (Lot# RAT316311)<br>HQC2: 164 pg/mL (Lot# RAT316324)   | <b>Spiked EQC3</b> 312 pg/mL (Lot#TCB230321-01)              |
| <b>Material used for buffer quality controls (bQCs) and concentration</b>     | <b>ACTH Stock</b>        | Rat Stress Hormone Standard & Kit Controls                             | Canine Pituitary Expanded Panel Standard & Kit Controls      |
|   | <b>Surrogate Matrix</b>  | Assay Buffer   | Assay Buffer   |
|   | <b>LLOQ1 QC</b>          | 1.37 pg/mL (Recombinant Spike)   | 10.3 pg/mL (Recombinant Spike)                               |
|   | <b>LLOQ2 QC</b>          | 4.12 pg/mL (Recombinant Spike)   | 3.43 pg/mL (Recombinant Spike)                               |
|   | <b>BQC1</b>              | 21.5 pg/mL (Kit Control 1)   | 47.5 pg/mL (Kit Control 1)                                   |
|   | <b>BQC2</b>              | 108 pg/mL (Kit Control 2)  | 388 pg/mL (Kit Control 2)                                    |
|   | <b>BQC3</b>              | NA   | 1000 pg/mL (Recombinant Spike)                               |
|   | <b>ULOQ</b>              | 1000 pg/mL (Recombinant Spike)   | 2500 pg/mL (Recombinant Spike)                               |
| <b>MRD</b>  | <b>MRD</b>               | 4-fold   | 2-fold   |
|   | <b>Sample Volume</b>     | 20 µL  | 40 µL  |
| <b>Data Acquisition</b>   | <b>Equipment</b>         | BioRad Luminex 200   |  |
|   | <b>Program</b>           | BioRad Bioplex Manager version 11.0                                    |  |
|   | <b>Program</b>           | Watson™ LIMS Version 7.4.1   |  |
| <b>Data Analysis</b>  | <b>Regression</b>        | 4-parameter (Logistic Auto-estimate)                                   |  |
|   | <b>Weighting</b>         | 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 |
|----------------------------|---------------------------|--|---------------------------------|
|                            | <b>Catalog Number</b>     | RSHMAG-69K                                       | CANPIT-96K                      |
|                            | <b>Lot Number</b>         | 3931433  | 3990441                         |
|                            | <b>Expiration Date</b>    | 30 Apr 2024                                      | 31 Jan 2024                     |
|                            | <b>Storage Conditions</b> | 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; <sup>a</sup> %RE values are "0.0" as the Inter-batch runs are used to determine the nominal concentration of the matrix QCs. | <b>QC</b>   | <b>%CV</b> | <b>%RE</b>        | <b>%TE</b> | <b>QC</b>   | <b>%CV</b> | <b>%RE</b>        | <b>%TE</b> |
|   |   | 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% <sup>a</sup> | 8.2%       | EQC1  | 8.0%       | 0.0% <sup>a</sup> | 8.0%       |
|   |   | LQC2  | 8.8%       | 0.0% <sup>a</sup> | 8.8%       |   |            |                   |            |
|   |   | MQC1  | 3.1%       | 0.0% <sup>a</sup> | 3.1%       | EQC2  | 10.4%      | 0.0% <sup>a</sup> | 10.4%      |
|   |   | MQC2  | 14.0%      | 0.0% <sup>a</sup> | 14.0%      |   |            |                   |            |
|   |   | HQC1  | 4.2%       | 0.0% <sup>a</sup> | 4.2%       | Spiked EQC3   | 4.5%       | 0.0% <sup>a</sup> | 4.5%       |
|   |   | HQC2  | 5.2%       | 0.0% <sup>a</sup> | 5.2%       | BQC1  | 4.0%       | 0.2%              | 4.2%       |
|   |   | BQC1  | 5.3%       | -0.5%             | 5.8%       | BQC2  | 4.6%       | 2.1%              | 6.7%       |
| BQC2  | 2.6%  | -1.9%   | 4.5%       | BQC3              | 2.1%       | -3.3%   | 5.4%       |                   |            |
| BQC3  | NA  | NA  | NA         | ULOQ              | 7.5%       | 10.4%   | 17.9%      |                   |            |
| ULOQ  | 10.6%   | -0.4%   | 11.0%      |                   |            |   |            |                   |            |
| 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. |            |                   |            | • Parallelism was not observed in low-endogenous tested lots due to samples measuring BQL.                          |            |                   |            |
|   |   | • Samples may be diluted 4, 8, or 16-fold.  |            |                   |            | • Two incurred samples with endogenous ACTH were tested. Both lots were parallel at the 2-fold and 4-fold dilution. |            |                   |            |
| 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 3.43 pg/mL test was consistent between normal lots (93.6%).                                     |            |                   |            |
|   |   | • % Recovery at the 145 pg/mL test was consistent between normal lots (mean of 74.6%).          |            |                   |            | • % 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.