Poster Number: D1-P04

COMPARISON OF DIFFERENT ASSAY KITS FOR DOG SERUM PARATHYROID HORMONE (PTH) MEASUREMENT

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NOVEL ASPECTS

1. In light of the newly issued FDA Bioanalytical Method Validation for Biomarkers guideline, our study emphasizes the importance of confirming the method specificity for the intended analyte using a fit-for-purpose approach.
2. In this study, we conducted a comparison of commercial PTH ELISA kits from two different vendors. The substantial difference in the stimulated dog serum samples between the two kits highlighted the limitation of the current kit qualification procedure and underscored the necessity of incorporating additional standards in specificity assessments. Based on our results, we then further investigated the prevalent designs of PTH quantification assay formats and offer recommendations for the selection of PTH measurements kits.

INTRODUCTION

PTH, synthesized by the parathyroid gland, is an 84-amino acid single-chain peptide crucial for calcium homeostasis. Its precursor, the 115-amino acid pre-proPTH, undergoes proteolytic cleavage to form the intermediate 90-amino acid proPTH, which is then processed into the biologically active PTH (1-84). PTH is an important biomarker for chronic kidney disease and parathyroid disorders.

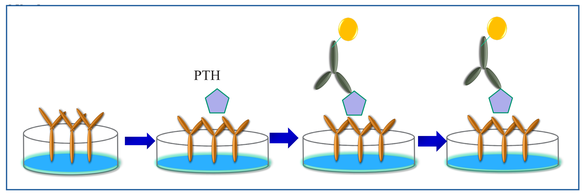
There are a variety of commercial PTH ELISA kits available for research purpose. The second generation of the kit often bears the misleading name of “intact”, also detects the 7-84 metabolite of PTH, whereas the third generation, referred to as “whole” or “bioactive” assay, utilizes the detection antibody targeting the 1-4 region. The applicability of different kits in measuring PTH in real samples has not been thoroughly investigated. In this study, we compared two commercial PTH ELISA kits. Both kits showed acceptable accuracy and precision during qualifications; however, there were differences in the PTH level in dog serums.

Our results underscore the limitations of the current kit qualification process and emphasize the importance of including additional standards in specificity measurements. Based on our findings and the design of popular PTH quantification assay formats, we provide recommendations for additional assay qualification to improve the reliability and accuracy of PTH assays.

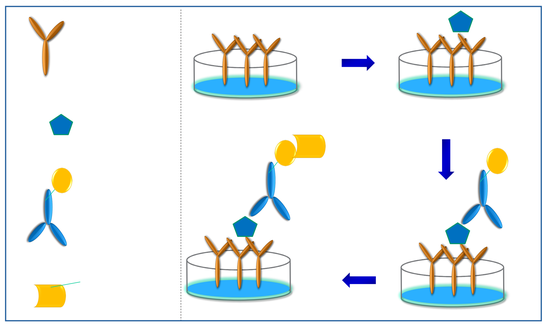
METHODS

1. Principle:

Item	Kit A	Kit B
Plate	Streptavidin-ELISA plate	High-bind plate
Coating reagent	Biotin-anti-human PTH antibody	Anti-canine PTH antibody
Reference	Canine PTH	Canine PTH
Detection reagent	HRP conjugated anti-human PTH antibody	Biotin-anti-canine PTH antibody and SA-HRP



Kit B Workflow:



2. Sample Collection:

- Serum samples were collected from healthy dogs.
- Samples were stored at <-60°C until analysis.

3. Experiment:

Dog serum:
Serum samples were collected from 70-month-old male beagle dogs from WuXi AppTec animal facility, with approval from the Institutional Animal Care and Use Committee. Collected serum was stored at ≤ -60°C until PTH analysis.

Analysis procedure:
The experiment was performed following the manufacturer’s protocols. Absorbance was measured using Molecular Device SpectraMax M2e. PTH level was back-calculated using a 4-parameter logistic curve fitted to kit standards.

Qualification of parameters included:
Assay range, the endogenous level from twenty individual serum from naïve canines, minimum required dilution factor (MRD) determination through selectivity and parallelism, intra/inter precision, short stability (including 8 hours at 2-8°C and at room temperature, 5 freeze/thaw cycles) and long term stability (3 months), cross-reactivity of the two kits.

RESULTS

1. Assay Range

Item	Assay Range in Buffer
Kit A	10-2000 pg/mL
Kit B	3.125-200 pg/mL

2. Endogenous Level

Select 20 serum samples from naïve canines and test the basal levels using two different kits. The specific results are as follows:

Item	Basal Level
Kit A	10-55 pg/mL
Kit B	~20-130 pg/mL

3. Dilution Linearity

Item	Parallelism	Selectivity	Minimum Dilution Factor
Kit A	Pass	Pass	1:2
Kit B	Pass	Pass	1:2

4. Quality Control

Item	QC
Kit A	Zero blank with 0 pg/mL human PTH HQC: 650.039 pg/mL LQC: 134.978 pg/mL
Kit B	No quality control samples

5. Precision

The acceptance criteria: CV ≤20%

Item	Intra-assay CVs	Inter-assay CVs
Kit A	0.5%-17.0%	8.3%-8.7%
Kit B	0.1%-10.9%	0.9%-2.0%

6. Stability

Item	Bench-top stability	Long-term stability
Kit A	Stable for at least 8 hours at 2-8 °C and room temperature, and after 5 freeze/thaw cycles	Stable for at least 3 months
Kit B	Stable for at least 8 hours at 2-8 °C and room temperature, and after 5 freeze/thaw cycles	Stable for at least 3 months

7. Kit Cross-Reactivity Analysis

- Using Kit A to detect the reference of Kit B resulted in no signal value.
- Using Kit B to detect the reference of Kit A also resulted in no signal value.

8. Stimulated Samples Analysis

Sample Description	Kit A Results (pg/mL)	Kit B Results (pg/mL)
Naïve Sample	63.580	51.8761
Pretest Sample	22.651	38.1802
Stimulated Sample-1	33.624	42.6778
Stimulated Sample-2	38.299	40.7606
Stimulated Sample-3	341.428	41.0632
Stimulated Sample-4	453.300	40.8979
Stimulated Sample-5	351.532	38.2207
Stimulated Sample-6	207.300	34.3217

DISCUSSION

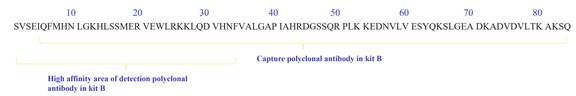
Dog PTH (1-84) Sequence Information

SVSEIQFMHN LGKHLSSMER VEWLRKKLQD VHNFVALGAP IAHRDGSSQR PLK KEDNVLV ESYQKSLGEA DKADVDVLTK AKSQ

After a thorough analysis of the characteristics of the substances detected by the kits, it was found that although both Kit A and Kit B can theoretically detect intact PTH, the antibodies target different regions of PTH and exhibit varying affinities. Kit A tends to detect intact PTH and fragments that contain sequences from both 1-34 and 39-87 regions, whereas Kit B also detects intact PTH but may have a higher affinity for the 1-34 region of PTH.

Kit A:

Kit B:



Kit A is designed to detect the intact 1-84 PTH form, and exhibits cross-reactivity with an inactive C-terminal fragment, specifically the 7-84 from but not with the 1-34 fragment.

In contrast, in addition to the intact PTH, Kit B has a high affinity for the PTH 1-34 fragment. This discrepancy might lead to significant differences in the detection results of the same stimulated sample. The short half-life of PTH leads to the rapid metabolism of intact PTH into bioactive PTH and various longer-lasting inactive fragments in circulation, necessitating careful selection of assay kits based on the specific PTH forms being studied.

CONCLUSIONS

1. **Specificity Differences**: Kit A targets intact 1-84 PTH with cross-reactivity to inactive C-terminal fragments (e.g., 7-84), whereas Kit B strongly binds both intact PTH and the bioactive N-terminal fragment (1-34). The kits specificity difference contributes to the significant discrepancy in the result, while other possible reasons can’t be excluded.
2. **Assay Selection Critical for Accuracy**: The short half-life of intact PTH and its rapid metabolism into fragments (e.g., 1-34 or 7-84) necessitate careful kit selection based on the target PTH form (intact vs fragments) to ensure biologically relevant data.
3. **Methodological Implications**: Cross-reactivity and fragment affinity differences between kits highlight the risk of misinterpretation in PTH studies, emphasizing the need for validation against the specific PTH forms under investigation.

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