



**LifeAssays AB**

Ideon Science Park

Scheelevägen 19F

223 70 Lund

Sweden

Att.: Kirstin Kriz, Head of Research & Development

**Commissioned Research report: Method evaluation and method comparison of LifeAssays canine CRP point-of-care system**

Please find enclosed the final report of the method evaluation and method comparison of LifeAssays® canine CRP point-of-care (POCT) system according to the commissioned research agreement.

Yours sincerely,

Mads Kjelgaard-Hansen, DVM, PhD, Assoc. professor  
Department of Small Animal Clinical Sciences  
Faculty of Life Sciences  
University of Copenhagen  
Gronnegaardsvej 3 st.  
DK-1870 Frederiksberg C  
Denmark  
+45 35333873 (Phone)  
+45 35332947 (Fax)  
[mjkh@life.ku.dk](mailto:mjkh@life.ku.dk) (e-mail)

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DEPARTMENT OF SMALL ANIMAL  
CLINICAL SCIENCES  
VETERINARY CLINICAL PATHOLOGY

3 GRONNEGAARDSVEJ  
DK-1870 FREDERIKSBERG C  
DENMARK

PHONE +45 3533 3873  
FAX +45 3528 2947

MJKH@LIFE.KU.DK

## **Evaluation of the LifeAssays® Canine CRP System for routine canine C-Reactive Protein (CRP) measurements for clinical purposes**

### ***Abstract***

The aim of the present study was to evaluate the analytical performance and reliability of the LifeAssays® Canine CRP System – a new magnetic permeability based assay for diagnostic measurements of canine C-reactive Protein (CRP). The performance was evaluated by assessment of components of analytical performance (intra- and interassay imprecision, detection limit and markers of inaccuracy; spike and recovery and linearity under dilution). Method comparison to a validated reference method was performed to further identify and characterize possible bias of clinical importance.

The analytical performance of the LifeAssays® Canine CRP system was acceptable with intra- and interassay coefficients of variations of 5.0-13.6% and 4.5-16.7%, respectively with the highest variations observed for measurements of CRP in serum with concentrations in the normal range (below the clinical decision level). Recovery of 60-89% and acceptable linearity under dilution were observed. Although a discrepancy exceeding the combined imprecision in the measured CRP levels was observed when compared to the reference method; a good agreement on diagnostic classification was present, suggesting the discrepancy to be of low clinical significance. The Life Assays® Canine CRP system performed reliably in determination of canine serum CRP for clinical purposes in a POCT setting, however, direct comparison of results with results from other systems should be done with caution.

### ***Abbreviations***

CRP: C-Reactive Protein, CI: Confidence interval, CV: Coefficient of variation, SD: Standard Deviation, TIA: Turbidometric Immunoassay.

### ***Introduction***

C-reactive protein (CRP) is a major acute phase protein in dogs. The applicability of routine measurements for diagnostic purposes has been well documented (Kjelgaard-Hansen, 2004) and additional studies are continuously performed further demonstrating this applicability (Dabrowski et al., 2007; Gebhardt et al., 2009; Griebisch et al., 2009).

Several methods have been developed for measurements of canine CRP. Among others, methods as Enzyme-linked Immunosorbent-assay (ELISA), Enzyme linked sorbent assay (ELSA), electroimmunoassay and Time-resolved Immunofluorometric Assay (TR-IFMA) have proven useful as methods for CRP measurements for research purposes (Caspi et al., 1984; Deegan et al., 2003; Kjelgaard-Hansen et al., 2003b; Parra et al., 2006). To be applicable for routine diagnostic purposes the methods need to be quick and easy performed and a human turbidometric immunoassay (TIA) is validated for quantitative measurements for diagnostic purposes at veterinary laboratories (Kjelgaard-Hansen et al., 2003a). A reversed passive latex agglutination test is validated for semi quantitative measurements of CRP in clinical settings (Kjelgaard-Hansen et al., 2008), but near-patient methods for quantitative measurements of canine CRP are

still lacking and are apparently necessary for appropriate clinical use (Kjelgaard-Hansen and Jacobsen, 2010). Magnetic permeability based assays have proven useful for near-patient diagnostic measurements of CRP in human medicine (Kriz et al., 2005). The LifeAssays® Canine CRP system is a new Magnetic permeability based assay developed for measurements of canine CRP and initial studies have given promising results of the performance of this assay (Ibraimi et al., 2009).

The aim of the present study was to evaluate the analytical performance and reliability of the LifeAssays® Canine CRP system in a routine clinical setting. The present work consists of an assessment of imprecision, detection limit, markers of inaccuracy and a method comparison, comparing the CRP measured by LifeAssays® Canine CRP system and an established and validated reference method applied routinely for clinical purposes.

The study was conducted as commissioned research.

### ***Materials and methods***

#### *CRP analysis*

The LifeAssays® Canine CRP system (lot E-963 and E-964) was used for measurements of canine CRP according to the recommendations from the manufacture (LifeAssays, IDEON Science Park, Lund, Sweden). Measurements were compared to measurements of CRP of the same samples run in parallel on a reference method; a Turbidometric Immunoassay previously validated for diagnostic use in dogs (Kjelgaard-Hansen et al., 2003a), calibrated and controlled species-specifically (Kjelgaard-Hansen et al., 2004; Kjelgaard-Hansen, 2010).

#### *Serum samples*

Serum samples from 47 client owned dogs was included in the study. All samples were obtained for diagnostic purposes and received as such at the Central Laboratory, Dept. of Small Animal Clinical Sciences, Faculty of LIFE Sciences, University of Copenhagen, Denmark in the period of January to November 2010. Serum was prepared by centrifugation (2000g, 5 minutes) of blood samples after clot formation. The serum samples were stored in plastic vials at -20°C until analysis. Samples were only thawed when needed for analysis in order to limit the number of freeze-thaw cycles.

#### *Assay characteristics of the LifeAssays® Canine CRP system*

Three pools of canine serum were established – containing different levels of CRP within the analytical spectrum of the LifeAssays® Canine CRP system – one pool below the clinical decision level used at the laboratory (<20 mg/L), one pool with a CRP content near the decision limit (40-45 mg/L) and one pool with a high level of CRP (150-180 mg/L).

The intra-assay imprecision was assessed by replicate measurements (n=15) of each of the 3 pools within the same day. The inter-assay imprecision was assessed by replicate measurements (n=8) of each pool across days.

Inaccuracy was investigated linearity under dilution and by spike and recovery - observations. For the study of linearity under dilution duplicate determinations of CRP were made after dilution of a serum pool with a high content of CRP diluted to hold 0%, 10%, 20%, 30%, 40%,

50%, 60%, 70%, 80%, 90% and 100% of the original concentration of CRP using distilled water as diluents.

The spike and recovery study was performed by spiking the low serum pool with purified canine CRP (LifeDiagnostics, US) to concentrations of 612 mg/L, 194 mg/L and 88 mg/L, respectively.

Detection limit was assessed based on data from 5 replicate measurements on samples without canine CRP (distilled water).

Method comparison was performed to further identify and characterize possible inaccuracy of clinical relevance by measuring CRP by means of the LifeAssays® Canine CRP system and the reference method in parallel in 40 samples of canine serum covering the full spectrum of CRP concentrations most frequently encountered in clinical settings.

#### Statistical Analysis and performance criteria

Arithmetic means, SDs, Intra- and interassay CVs were calculated using routine descriptive statistical procedures. Performance criterion for imprecision was derived from data on biological variation of canine CRP (Kjelgaard-Hansen et al., 2003c) as recommended (Kenny et al., 1999; Kjelgaard-Hansen and Jensen, 2010), and thus set to  $CV_{max}=12.2\%$ .

Assessment of linearity under dilution was performed by ordinary least-square linear regression analysis. Performance of linearity under dilution was assessed acceptable if regression line did not deviate significantly from a slope of 1, a Y- intercept of 0 and data fitted a linear regression model (tested by Run's test).

Recovery was assessed by calculation of the fraction of recovered purified canine CRP, acceptable if within 80%-120% recovery.

Method comparison was performed by Deming regression, and criteria for acceptable agreement were a line not deviating significantly from a slope of 1, a Y- intercept of 0. To assess whether possible discrepancies exceeded that explainable by mere combined imprecision a Bland Altman Plot analysis was done and differences between measurements were related to the lines representing  $0 \pm$  the 95% confidence interval of combined inherent imprecision (Jensen and Kjelgaard-Hansen, 2006). The combined inherent imprecision was calculated from the imprecision observed in the present study and the imprecision for the reference method (Kjelgaard-Hansen et al., 2003a).

Level of significance was set to  $P<0.05$ .

## **Results**

### Imprecision

The observed intra- and inter-assay imprecision ranged from CVs of 5.0 to 13.6% and 4.5-16.7%, respectively, with the highest CVs observed in serum samples with CRP concentrations below the clinical decision limit. Only Lot E-963 exceeded the performance criteria of  $CV_{max}=12.2\%$ .

Table 1. Intra- and inter-assay imprecision in the determination of C-reactive Protein (CRP) concentration after replicate measurements of 3 pools of canine serum.

	No. Samples	Mean [95% CI] (mg/L)		SD (mg/L)		CV	
		Lot E-963	Lot E-964	Lot E-963	Lot E-964	Lot E-963	Lot E-964
Intraassay	15	25.5 [23.6;27.5]	26.7 [25.4;28.1]	3.48	2.49	13.6%	9.3%
	15	42.2 [40.4;44]	48 [46.7;49.3]	3.26	2.39	7.7%	5.0%
	15	177.9 [172.3;183.6]	180.8 [170.4;191.2]	10.2	18.8	5.8%	10.4%
Interassay	8	27.5 [23.7;31.3]	31.3 [28.1;34.4]	4.60	3.81	16.7%	12.2%
	8	43.0 [39.7;46.2]	45.8 [42.6;48.9]	3.85	3.81	9.0%	8.3%
	8	189.0 [178;200]	189.7 [180.6;198.7]	13.2	8.62	7.0%	4.5%

CV; coefficient of variation, SD; Standard Deviation, CI; confidence interval.

### Inaccuracy

The investigation of inaccuracy by linearity under dilution resulted in a linear regression equation in which (x,y)= (expected level according to dilution of serum pool, measured level) (Figure 1). Regression line slope did not differ from 1, the intercept did not differ from 0 and the data fitted a linear model (Runs test). Thus, no significant internal inaccuracy was detected.

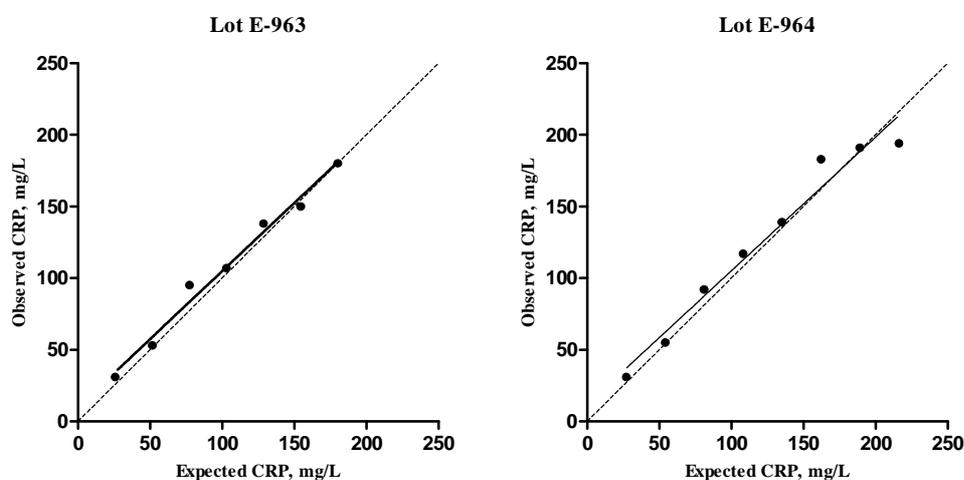


Figure 1. Graphic illustration of the linearity under dilution. Values of expected C-Reactive Protein (CRP) concentration are calculated from the CRP-value measured at 70% dilution of original pool containing high concentration of canine CRP. Black line represents regression line. Broken line represents line of agreement (Y=X). Regression line slope [95% confidence interval], Y-intercept [95% CI] and P-value (Runs test) were 0.95 [0.81;1.09], 10 [-5.7;25.7], 1.0 and 0.93 [0.76;1.1], 11.9 [-11.6;35.4], 0.14 for lot E-963 and E-964, respectively.

The results of the investigation of spike and recovery are given in table 2, showing acceptable recovery rates (85.3-89%) for all measurements except one (60% for low spike of lot E-964). The exact recovery rate could not be calculated when samples were spiked to concentrations

above the measuring limits of the assay, but data indicated that a clinically important prozone (hook) effect is not present up to CRP concentrations of 610 mg/L.

Table 2 Observed recovery of canine CRP in serum samples spiked with purified canine CRP. Baseline level of serum sample was 25.5mg/L and 26.7mg/L for lot E-963 and lot E-964, respectively.

	Low		Medium		High	
	Lot E-963	Lot E-964	Lot E-963	Lot E-964	Lot E-963	Lot E-964
Expected CRP (mg/L)	85.7	87.9	192.2	193.8	610.6	611.6
Observed CRP (mg/L)	77	64	169	176	>210	>210
Recovery	85.3%	60%	86%	89%	-	-

#### Detection limit

Samples without canine CRP (n=5) were all observed to be below the internal analytical cut-off of the assay (10 mg/L) sustaining this as the functional detection limit of the assay.

#### Method comparison

Method comparison between the LifeAssays POCT system and the reference method by Demings regression (Figure 2) revealed no systematic inaccuracy; Y-intercepts [95% CI] of 2.8 [-11.8; 17.4] and 6.84 [-8.26;21.9] for lot E-963 and lot E-94, respectively. Also no proportional inaccuracy was observed; slope [95% CI] of 0.88 [0.7; 1.06] and 0.89 [0.7; 1.07], for lot E-963 and lot E-964, respectively.

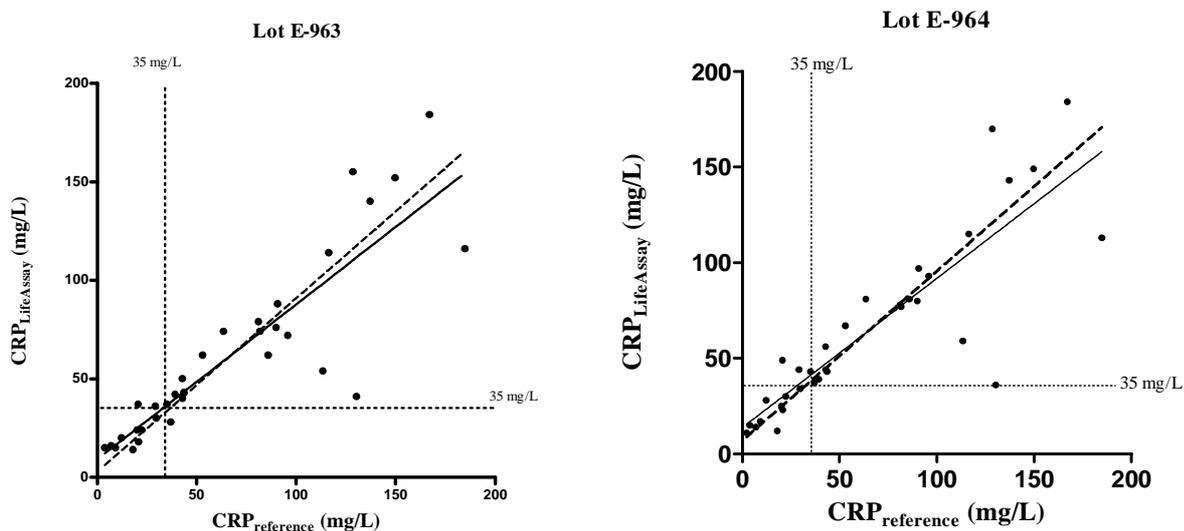


Figure 2 Regression analyses for C-reactive protein (CRP) concentrations in canine serum samples measured by LifeAssays® Canine CRP system and a reference method. Demings regression line (Black line). Line of agreement (broken line). Clinical decision limits given for the two assays (vertical and horizontal lines, respectively). Single observations (black circles).

However, for a significant proportion of single observations (14/40 and 18/40 for lot E-963 and lot E-964, respectively) the level of absolute discrepancy was observed to exceed that explainable by mere combined imprecision ( $CV_{\text{combined}}=9.4\%$ ) (Figure 3).

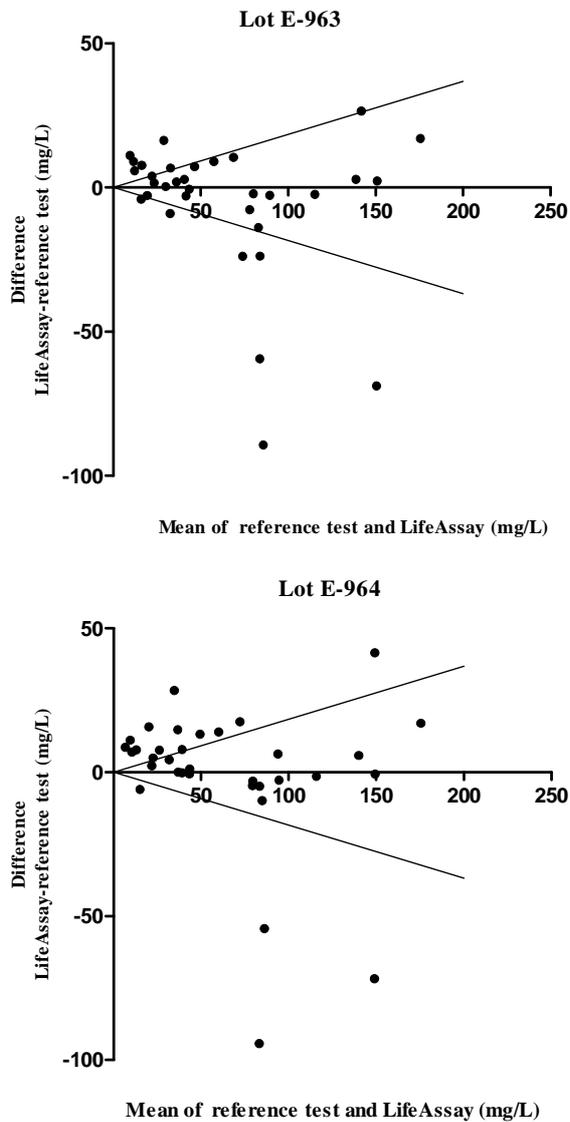


Figure 3: Bland Altman difference plot for C-reactive Protein (CRP) concentrations in canine serum samples measured by the LifeAssays® Canine CRP system and a reference method. Individual measurements (black circles). Limit for discrepancy explainable by mere combined imprecision (black lines).

The identified discrepancy is however of seemingly little clinical significance as a good agreement on diagnostic classification according to clinical decision limits was observed, with an overall agreement of 91 and 93% and a kappa of 0.79 and 0.86 for Lot E-963 and E-964, respectively. This agreement is illustrated in figure 2.

## ***Discussion***

The overall assessment of the LifeAssays® Canine CRP system demonstrated that the system performs acceptable for a point-of-care setting. The LifeAssays® Canine CRP system measured canine serum CRP concentrations at the clinical decision level with intra- and interassay imprecision acceptable for a POCT setting. Imprecision exceeding the general performance criteria was observed at a CRP concentration below the clinical cut-off, however as this is of less importance to the clinical use of the assay, this is most likely not of less clinical importance.

No signs of critical internal inaccuracy were detected. When measurements were directly compared to an established reference method for canine CRP measurements, neither systematic nor proportional discrepancy were detected, however a large proportion of discrepancies in single measurements exceeded the level explainable by mere combined imprecision. Important, this discrepancy did apparently not lead to a significant proportion of clinically important misclassifications. Direct comparison of results across assay systems should however only be made with great caution.

In conclusion, the LifeAssays® Canine CRP system seems to be a reliable method for diagnostic measurements of canine CRP in a POCT setting.

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