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Clinical study of canine tear lacritin as a treatment for dry eye

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Clinical Study of Canine Tear Lacritin as a Treatment for Dry Eye

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ABSTRACT

Keratoconjunctivitis sicca (KCS), the deficiency of tears also known as dry eye, is a prevalent disease that affects both humans and canines. The current treatment for dye eye, cyclosporine (Restatis®), only provides temporary relief, is often associated with discomfort and is inconsistently effective. Lacritin is a naturally occurring tear glycoprotein secreted from the human and canine lacrimal glands. It has been shown that lacritin stimulates basal tearing in rabbits when applied topically. This study characterized the amount and form of lacritin found in the tears of dogs with healthy and dry eyes—information which may be applied to the development of a lacritin-inspired therapeutic for humans and canines. In collaboration with the Virginia-Maryland College of Veterinarian Medicine, tear samples were collected from dogs being treated at the veterinary clinic and transported to JMU for analysis. At JMU, the lacritin and other proteins found in the canine tears were analyzed by indirect Enzyme-Linked Immunosorbent Assay (ELISA), SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and western blot. A total of 64 tear samples were analyzed with 32 samples from healthy dogs and 32 samples from dogs clinically diagnosed with dry eye. ELISA revealed that canines with KCS had a significant decrease in tear lacritin. Western blot analysis detected prominent bands in healthy tears at approximately 18 kDa (corresponding to monomeric canine lacritin) that were absent or faintly observed in tears from dry eye dogs. This study provides clinical data reinforces the hypothesis that lacritin replacement may be an effective therapeutic for dry eye.

INTRODUCTION

Background

Keratoconjunctivitis sicca (KCS), the deficiency of tears also known as dry eye, is a prevalent disease that affects both humans and canines. Symptoms include dry, burning, itchy, scratchy, stingy, or tired eyes (Merck Manual, 2003). Dry eye is the most common eye disease, affecting the quality of life of over 25 million Americans, especially women and the geriatric population (Seifert *et al*., 2012). Blepharitis is a complex inflammation of the eyelid associated with dry eye and meibomian gland dysfunction. Next to dry eye, it is one of the most common conditions seen by ophthalmologists. The current treatment for dye eye, cyclosporine (Restatis®), increases tear production, but only provides temporary relief. Additionally, some people have little or no effective reaction to the medication (Ofria *et al*., 2009). A proteomics study recently discovered certain tear proteins were selectively down-regulated in tears of patients suffering from chronic blepharitis and dry eye (Koo *et al*., 2005). Down-regulation of naturally occurring tear proteins may provide biomarkers for the diagnosis of ocular diseases and replacement therapeutic treatments for these disorders.

Lacritin is a naturally occurring human tear glycoprotein discovered and first characterized by Gordon Laurie at the University of Virginia as a novel secretion enhancing factor from the human lacrimal gland (Sangi *et al*., 2001). It is a prosecretory mitogen that consists of 119 amino acids and is 12.3 kDa in size produced by the lacrimal gland that exits the gland through acinar secretory granules and flows through ducts to the surface of the eye (Ma *et al*., 2008). It has been found that when lacritin is bound to corneal epithelial cells, it stimulates the lacrimal gland to produce tears (Sanghi *et al*., 2001). The down-regulation of lacritin has been found in patients with blepharitis (Tsai *et al*., 2006) and lacritin is the only prosecretory protein that is down-regulated in patients suffering from dry eye (Srinivasan *et al*., 2012). Preclinical animal studies have shown that recombinant human lacritin produced in *Escherichia coli* promotes basal tearing in rabbit eyes upon topical application (Samudre *et al*., 2011). Recently, a prototype clinical assay to quantify lacritin in human tear samples was developed (Seifert *et al*., 2012) and used to detect a lacritin-like protein in horse tears (Laurie *et al*., 2012). In addition, it was shown that a cleavage potentiated C-terminal fragment of lacritin is bactericidal against Gram positive

and Gram negative ocular pathogens (McKown *et al*., 2014). Taken together, these observations suggest that tear lacritin may be an influential ocular surface protector.

KCS is a common ophthalmic disease in dogs, with the recent literature citing a prevalence of 4% in the general canine population (Williams *et al.*, 2008). Canine KCS can occur for a variety of reasons, the most common being decreased aqueous tear production from immune-mediated inflammation of the lacrimal gland (Williams *et al.*, 2008). As such lifelong therapy is typically necessary.

A consortium of researchers from James Madison University (JMU), the University of Virginia (UVa), Walter Reed Army Medical Center, Eastern Virginia Medical School, and other institutions investigate lacritin as a potentially new human therapeutic for the treatment of dry eye. With support from the 4-VA Collaborative, a new JMU—Virginia-Maryland Regional College of Veterinary Medicine collaboration was initiated in 2012 to study the role of lacritin in canine dry eye. As a result, a lacritin-like protein was detected in canine tear samples by human anti-lacritin antibodies. With scale-up funding from 4-VA, the gene encoding the canine lacritin ortholog was cloned, sequenced, and a recombinant form was expressed and purified in *E. coli*. Antibodies specific to canine lacritin were produced and a canine Enzyme-Linked Immunosorbent Assay (ELISA) was developed. Preliminary results of this assay have revealed that canine lacritin is down-regulated in tears from dogs with dry eye.

Summary of Previous Work

Summary of Work Accomplished by Alan C. Tate, Honors Thesis, 2014

Tear samples collected at Virginia-Maryland Regional College of Veterinary Medicine were shipped to JMU for analysis. Total protein was eluted from the wicks by means of centrifugation and total protein concentrations were determined by a bicinchoninic acid (BCA) assay. An ELISA, previously developed at JMU to detect human lacritin, was used to quantify lacritin in dog tears. Recombinant human lacritin was used as a protein standard in the assay. Of the two anti-lacritin human polyclonal antibodies tested, the one directed towards the N-terminus was the most effective at detecting canine lacritin in the samples. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blots were performed on the canine samples using the human antibodies. Forms of lacritin similar to those observed in human tears were observed (Figure 1).

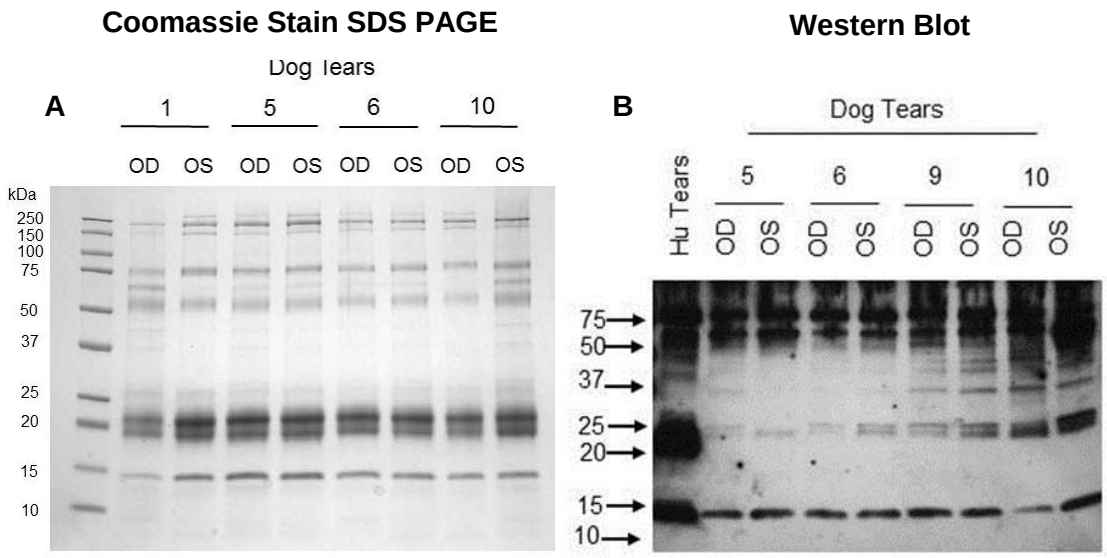


Figure 1. SDS-PAGE and western blot analysis of canine tears. (A) Tear samples 1, 5, 6, and 10 were separated by SDS-PAGE and visualized with Coomassie blue stain. A molecular weight standard is shown on the far left of the gel. Approximate band sizes are labeled. (B) Tear samples 5, 6, 9 and 10 were separated by SDS-PAGE, transferred to nitrocellulose, incubated with anti-N-terminal hlacritin antibodies, and detected by chemiluminescence. "Hu Tears" refers to a control human tear sample. "OD" and "OS" refer to samples taken from the right versus left eye from an individual, respectively (Tate, Honors Thesis 2012).

In order to make recombinant canine lacritin, total RNA was extracted from normal canine lacrimal glands, reverse transcribed (using gene-specific primers?) and PCR amplified. The resulting cDNA was cloned into the bacterial expression vector, pTYB2. Recombinant canine lacritin was expressed in *E. coli* and purified by chitin affinity column and diethyl-aminoethyl (DEAE) chromatography (Figure 2).

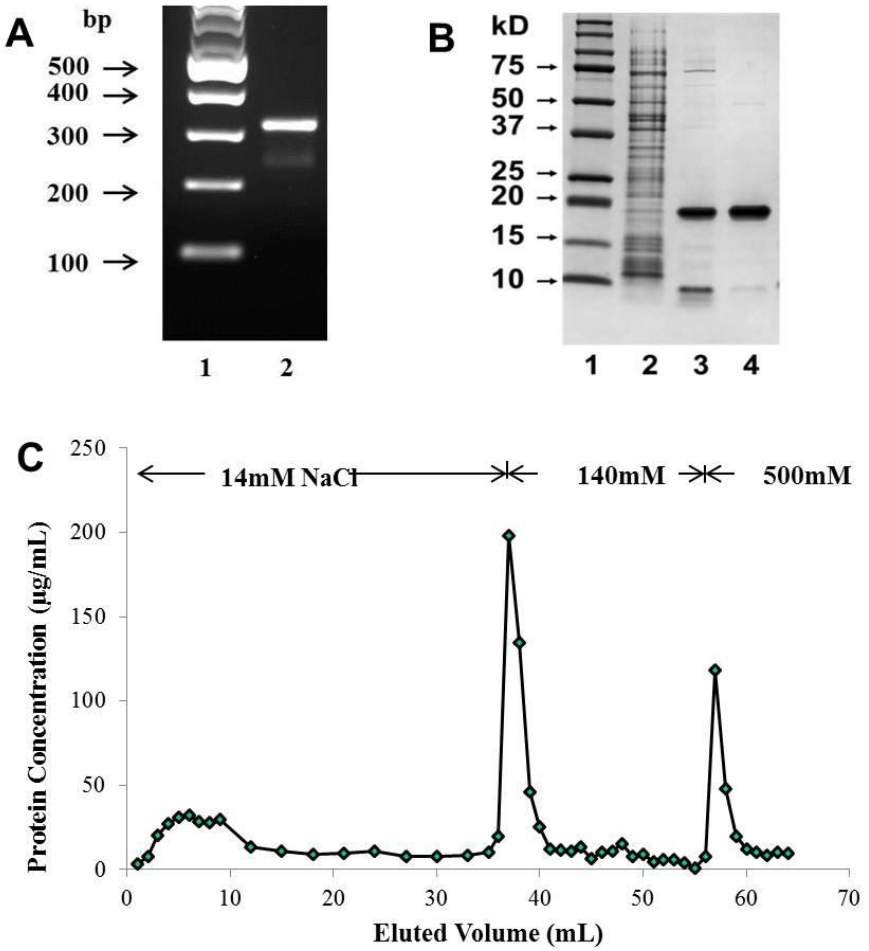


Figure 2. RT-PCR of canine lacritin gene, purification of recombinant canine lacritin, and DEAE chromatography elution profile. (A) Agarose DNA gel of the RT-PCR product. Lane 1 contains the 100 bp DNA ladder, with fragment sizes indicated. Lane 2 contains the amplified PCR product of the canine lacritin cDNA. (B) SDS-PAGE gel of 18 kD purified recombinant canine lacritin. Lane 1 contains the molecular weight standards, lane 2 contains the cleared cell lysate prior to chitin affinity chromatography, lane 3 contains the fraction extracted after chitin affinity chromatography, and lane 4 contains the eluted sample from the DEAE Sepharose column. (C) DEAE chromatography elution profile of recombinant canine lacritin. Following chitin affinity chromatography, protein fractions were pooled, dialyzed in 14 mM NaCl PBS, and loaded onto a DEAE sepharose column equilibrated with 14 mM NaCl PBS, pH 7.4. The column was eluted with 140 mM PBS and 500 mM NaCl PBS cuts. Protein concentrations of each fraction were determined using Pierce™ BCA Protein Assay Kit (Tate, Honors Thesis 2012).

Summary of Work Accomplished by Alison M. Enghauser, Honors Thesis, 2015

Anitbodies directed against the N- terminus of canine lacritin were produced and purified. The N-terminus peptide of canine lacritin EGDSSDPAPGAAAADPGGL was synthesized with >90% purity and conjugated to keyhole limpet hemocyanin/bovine serum

albumin (KLH/BSA) by Bio-Synthesis, Inc. (Lewisville, TX). Two New Zealand white rabbits, designated 6924 and 6925, were immunized and serum was collected over a 10 week period. Preimmune serum was collected before immunization and final antiserum was collected after 10 weeks. Protein A coupled with agarose beads (Bio-Rad Laboratories; Hercules, CA) was used to purify IgG antibodies from the anti-cLACRT NT antiserum. An indirect ELISA was used to titrate the anti-cLACT NT canine antibodies.

A preliminary study of canine tear lacritin was performed using the anti-cLACRT antibodies. Canine tear samples were obtained at the VA-MD Regional College of Veterinary Medicine at Virginia Tech, eluted off the wicks and total tear protein determined by a BCA assay. An indirect ELISA and western blot was used to quantify lacritin in healthy and dry eye canine tear samples. The ELISA was developed using 10 week anti-cLACRT NT PA purified antiserum (from rabbit 6924) as the primary antibody and the HRP-conjugated antibody as the secondary. A summary of lacritin concentrations in canine tear samples is shown in Figure 3, and western blot analysis in Figure 4. This work showed that the anti lacritin serum can successfully detect canine lacritn, as well as showing preliminary evidence that lacritin may be down regulated in the tears of canines with dry eye.

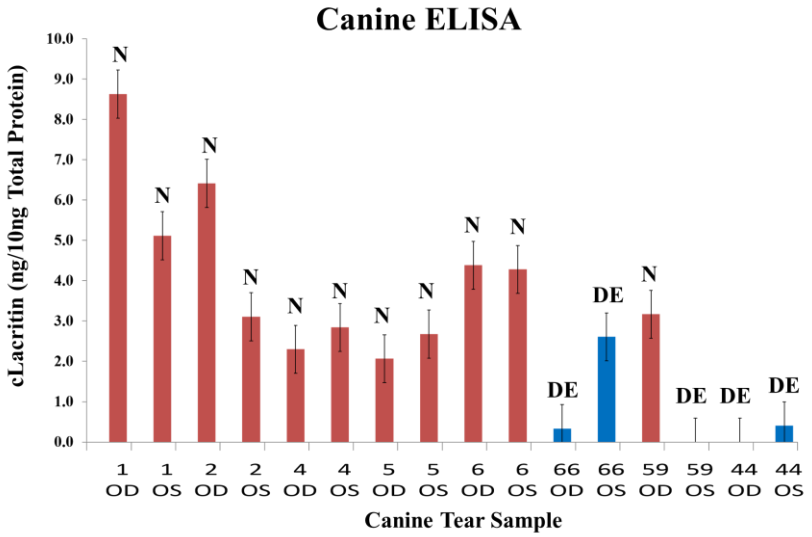


Figure 3. Lacritin concentrations in canine tears, as determined by indirect ELISA using anti-cLACT NT primary antiserum. "OD" and "OS" refer to samples taken from the right versus left eye from an individual, respectively. The origin of each sample is indicated: normal (blue, "N") or dry eye (red, "DE"). (Enghauser, Honors Thesis 2014).

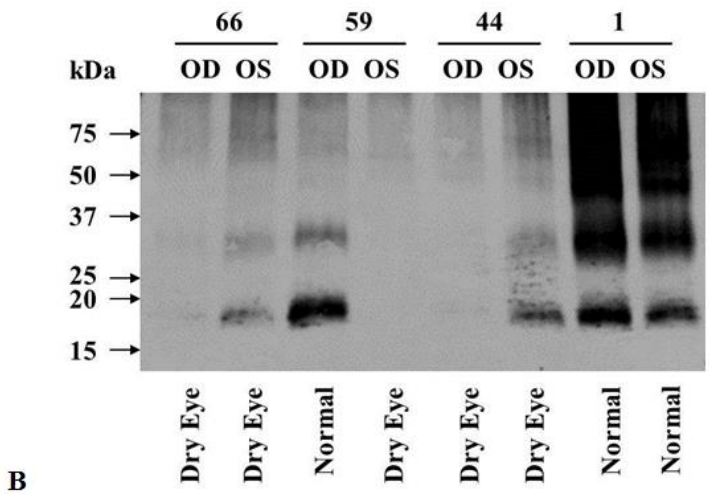


Figure 4. Western blot analysis of normal (1) and dry eye (66, 59, 44) tears from canine samples. A standard molecular weight ladder, with kilo-Dalton (kDa) band sizes indicated, is shown on the left. "OD" and "OS" refer to samples taken from the right versus left eye from an individual, respectively. The band corresponding to monomeric canine lacritin is seen at ~18 kDa (arrowhead). Bands with larger molecular weights represent cross-linked variations of lacritin . (Enghauser *et. al.,* 2014)

METHODS AND MATERIALS

Tear Collection and Elution

Dr. Julie Disney, a resident in Ophthalmology at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech, collected canine tear samples from 64 animals—32 from healthy and 32 dry eye dogs. Wicks were placed on each of the the canine's eyes to absorb tears. Samples were eluted by placing 30 µL of filter sterilized phosphate buffered saline (PBS) onto each wick, incubateding for 20 minutes, followed by centrifugation for 5 minutes at 13,000 rpm. Supernatants were collected as eluted tear samples and stored at -20° C.

Total Tear Protein Concentration

To determine the total tear protein concentration in each canine tear sample, the Thermo Scientific BCA Protein Assay Kit (Waltham, MA, USA) was used, as recommended by the manufacturer. Bovine serum albumin (BSA) Standards ranging from 20 to 2000 µg/mL and experimental samples were analyzed in duplicate in a 96-well microtiter plate following incubation for 30 minutes at 37° C. The plates were read at 570 nm in a spectrometer. The standard protein concentrations were graphed versus the absorbance values and a line of best fit was determined. Based on the equation of the line of best fit, each sample's total tear protein concentration was calculated.

Indirect ELISA Assay

The purpose of the indirect ELISA was to quantify the lacritin concentration in each canine tear sample. The indirect ELISA comprised of two components, the standard curve and the experimental samples. To create the standard curve, lyophilized recombinant canine lacritin (cLAC) was resuspended with 100 µL of deionized water, diluted with coating buffer (4.53 mL of 1.0 M $NaHCO_3$, 1.82 mL of 1.0 M Na_2CO_3, 93.65 mL dH_2O) and coated onto a 96-well microtiter plate with final concentrations of 0, 2, 4, 6, 8, 10, 12, 14, and 16 ng/mL. 100 µL of each standard was plated in triplicate.

The experimental samples were diluted with coating buffer to normalize the total tear protein to 0.5 μg/mL and 100 μL was added in triplicate to the plate. Samples were divided into eight sets of eight, such that each 96-well plate held eight samples. Each plate had a mix of both normal and dry eye samples, Duplicate plates were performed for ELISA analysis. All samples underwent the same procedure, as follows:

Plates were incubated overnight at 4°C, then washed three times with PBS/0.3% Tween-20 (PBST). Three hundred microliters of blocking buffer (1% w/v BSA) was applied to the wells. The plates were then incubated at 37°C for one hour. Following incubation, the plates were washed three times with PBST and then 100 μL of the primary antibody (6924 FB PANT) diluted 1:6400 in PBST was applied to each well. The plates were incubated again at 37°C for one hour. Next the plates were washed three times with PBST and 100 μL of the secondary HRP-conjugated goat anti-rabbit IgG antibody (Company or source) diluted 1:1200 in PBST was applied to each well. The plates were incubated for a third time at 37°C for one hour. Next the plates were washed three times with PBST and 100 μL of the o-phenylenediamine dihydrochloride (OPD) substrate was added. The OPD substrate was prepared by combining 6.0 mL of 0.1 M $C_6H_8O_7$, 6.5 mL of 0.2 M Na_2HPO_4, 12.5 mL dH_2O, 10 μL H_2O_2, and two OPD tablets (Acros Organics, Geel, Belgium). Plates were incubated in the dark for 10 minutes then read in the spectrophotometer at 415 nm. The absorbances of the known recombinant canine lacritin solutions were plotted against their concentration and a standard curve with a best-fit line was generated. The absorbance values of the experimental samples were used in the best-fit line to determine lacritin concentrations.

Antibody Specificity and Titrations

Final bleed N-Terminal Protein A-purified (PANT FB) antibodies from rabbit 6924 were titrated against known concentrations of recombinant canine lacritin to determine their specificity and optimal dilutions for the standard curve for the ELISA. Recombinant canine lacritin was diluted from 0.1 to 1000 ng/mL in coating buffer and 100 μL were applied to the wells of a 96-well microtiter plate. The 6924 PANT FB antibodies were diluted in PBST from 1:200 to 1:12800 and 100 μL were added to the wells and the

secondary HRP-conjugate goat anti-rabbit IgG antibody was diluted to 1:800 in PBST and 100 µL were added to each well.

A checkerboard titration was performed to optimize the dilutions of the primary and secondary antibody refine the dilutions determined by the titration and determine the optimal dilution for the secondary antibody. Canine lacritin was diluted from 1.5 to 20 ng/mL in coating buffer and plated. 6924 PANT FB primary antibodies were diluted to 1:400 in PBST and the secondary HRP-conjugated antibody was diluted from 1:200 to 1:12800 in PBST and applied to the plate and developed using the standard ELISA protocol.

SDS Polyacrylamide Gel Electrophoresis and Western Blots

Western blots were used to visually detect lacritin in the canine tear samples. Samples were diluted in PBS to 200 µg/mL and 20 µL was added to the wells. 4 µL of 6x loading dye was added. Ten microliter aliquots of the prepared samples were boiled for 5 minutes, and then loaded—along with 7 µL of the protein ladder (Precision Plus Protein Kaleidoscope Standards, BioRad)—into an Any kD™ Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad, ADD LOCATION) filled with Tris-glycine buffer and run at 200 volts until the samples reached the bottom of the gel. Proteins were then transferred onto a nitrocellulose membrane for western blotting. A Mini Trans-blot (COMPANY?), run for 60 minutes at 100 V, was used to transfer the samples from the gel to the membrane. The membranes were removed from the apparatus, wrapped in aluminum foil, and stored at 4°C overnight.

The blotted membranes underwent four 10-minute washes with PBST, and then incubated with the 1:1,000 dilution of primary antibody (6924 FB PANT) in PBST for one hour at room temperature. Next the membranes underwent four 10-minute washes in PBST, followed by a one hour incubation with the 1:5,000 dilution of secondary HRP-conjugated goat anti-rabbit IgG antibody in PBST. The membranes were then washed twice with PBST for 15 minutes each, followed by two 15-minute washes of PBS, then air-dried.

Blots were detected by chemiluminescence with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Rockford, IL), according to the manufacturer's recommendations. Membranes were soaked in substrate for one minute and covered in clear plastic wrap. Chemiluminescence was detected by X-ray film. Exposed film was

dipped in developer solution, deionized water, fixer solution and deionized water (30 seconds each) with continuous agitation.

RESULTS

Total Tear Protein Concentration

A BCA assay was used to determine the total protein concentrations of the dry eye and normal tear samples. Dry eye protein concentrations ranged from 367.1-7655 µg/mL, with a median of 1433.5 µg/mL, and an average of 2127 µg/mL. Normal protein concentrations ranged from 550.6-2485 µg/mL, with a median of 1323 µg/mL, and an average of 1379 µg/mL. All BCA assays had standard curves with R^2 values above 0.98. Figure 5 shows a representative standard curve. Results from the dry eye and normal samples are shown in Table 1 and Table 2, respectively. Raw data are shown in Appendix A.

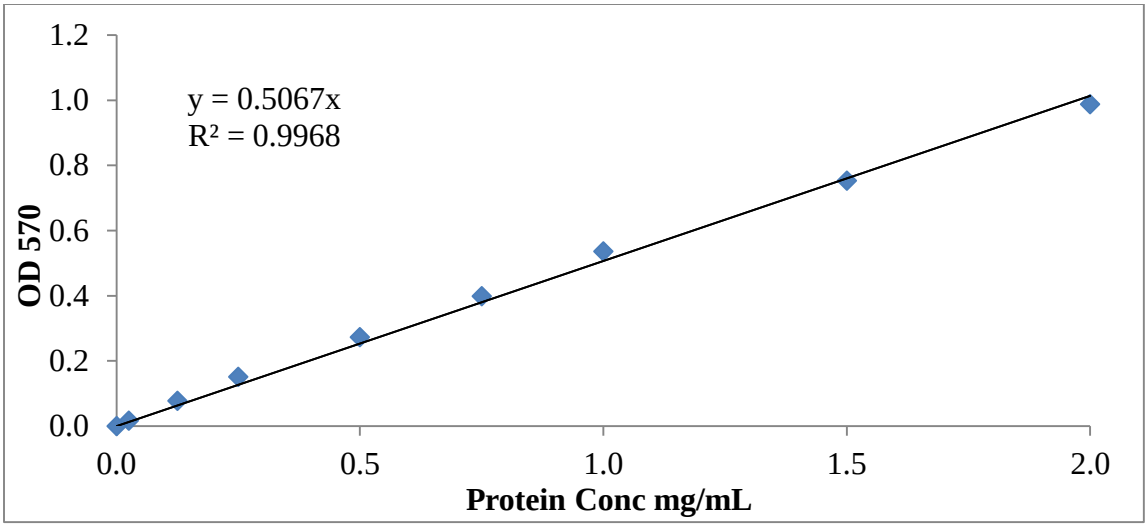


Figure 5. BCA Standard Curve. An example of a BCA assay standard curve.

Table 1. Summary of total tear protein in dry eye canines. The eye of origin (OD or OS) and concentration of each sample is indicated. Samples are listed in ascending order of average (left and right) total protein concentration.

Total Tear Protein in Dry Eye Canines

Sample ID	Eye	Total Protien (ug/mL)	Sample ID	Eye	Total Protien (ug/mL)
92935	OD	367.1	**134443**	OD	2148
	OS	503.3		OS	1165
137185	OS	833.2	**134581**	OS	1692
124839	OD	965.9	**93777**	OD	1485
	OS	783		OS	1970
122428	OD	552.7	**134918**	OD	2479
	OS	1360		OS	1067
134668	OD	1196	**134882**	OD	4807
	OS	1002		OS	1772
130826	OD	1089	**136361**	OD	3725
	OS	1137		OS	2968
135100	OD	1347	**134989**	OD	4055
131139	OS	1433		OS	2956
134564	OD	1434	**102059**	OS	4084
114039	OD	1967	**106997**	OD	7018
	OS	1073		OS	7655

Table 2. Summary of total tear protein in normal canines. The eye of origin (OD or OS) and concentration of each sample is indicated. Samples are listed in ascending order of average (left and right) total protein concentration.

Total Tear Protein in Normal Canines

Sample ID	Eye	Total Protien (ug/mL)	Sample ID	Eye	Total Protien (ug/mL)
121747	OD	550.6	**137481**	OD	1335
	OS	852.6		OS	1577
102059	OD	799.2	**137478**	OD	1217
135303	OD	704.8		OS	1737
	OS	1032	**119030**	OD	1675
135570	OS	1057		OS	1371
120039	OD	1175	**117503**	OD	1947
	OS	1091		OS	1265
129754	OD	1284	**114897**	OD	1844
	OS	1095		OS	1490
125091	OD	1722	**128415**	OD	1817
	OS	769.7		OS	1532
137477	OD	1201	**101803**	OD	1395
	OS	1311		OS	2015
131139	OD	1273	**135100**	OS	2485
87637	OD	1520			
	OS	1262			

Antibody Specificity and Titrations

A titration was performed on 6924 PANT FB to determine its specificity for recombinant canine lacritin and the optimal dilution for indirect ELISA. Dilutions of recombinant canine lacritin and the 6924 primary antiserum were created and used in a series of indirect ELISAs. The titration curve indicates that the antibodies detect lacritin at concentrations greater than 0.1 ng/mL (Figure 6).

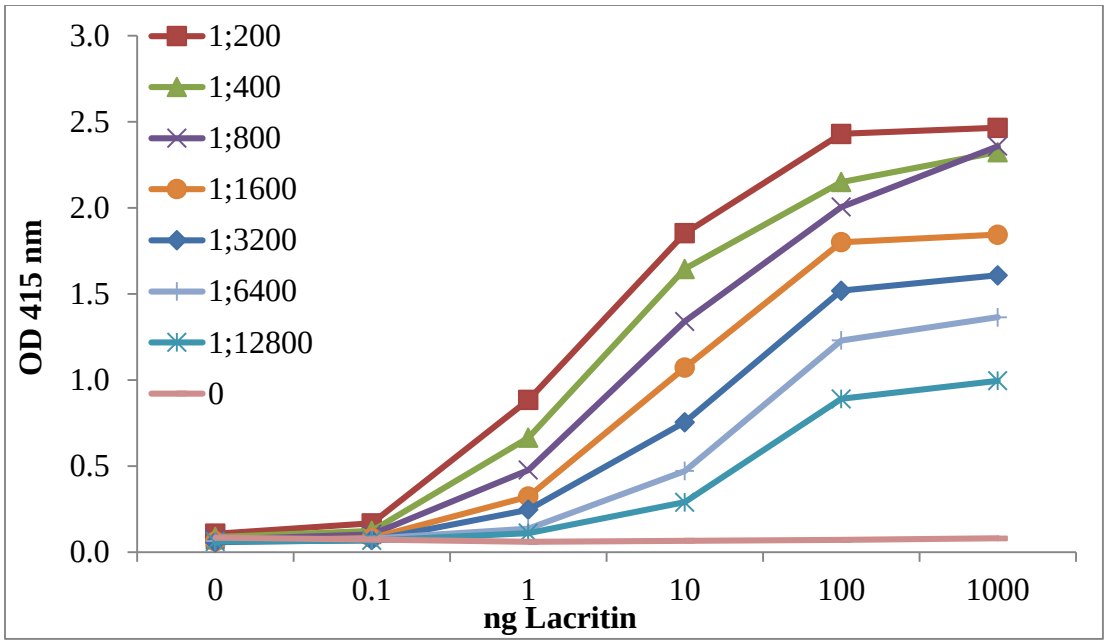


Figure 6. Titration of 6924 antibodies. A titration curve of antibody 6924 PANT FB where Lacritin was diluted from 0.1 to 1000 ng/mL and the primary antibody 6924 was diluted from 1:200 to 1:12800. The titration curve indicates that the antibodies can detect lacritin at concentrations greater than 0.1 ng/mL and that there is saturation of the assay at 100 ng.

A checkerboard titration was performed on the primary antibody 6924 PANT FB to determine the optimal dilutions of the primary and secondary for the standard curve (Figure 7). Lacritin was diluted to 1.5 to 20 ng/mL, the primary antibody 6924 PANT FB was diluted to 1:400 and the secondary antibody was diluted from 1:200 to 1:12800. Based on the titrations, the optimal dilution of the primary antibody was 1:6400 and the secondary antibody was 1:1200.

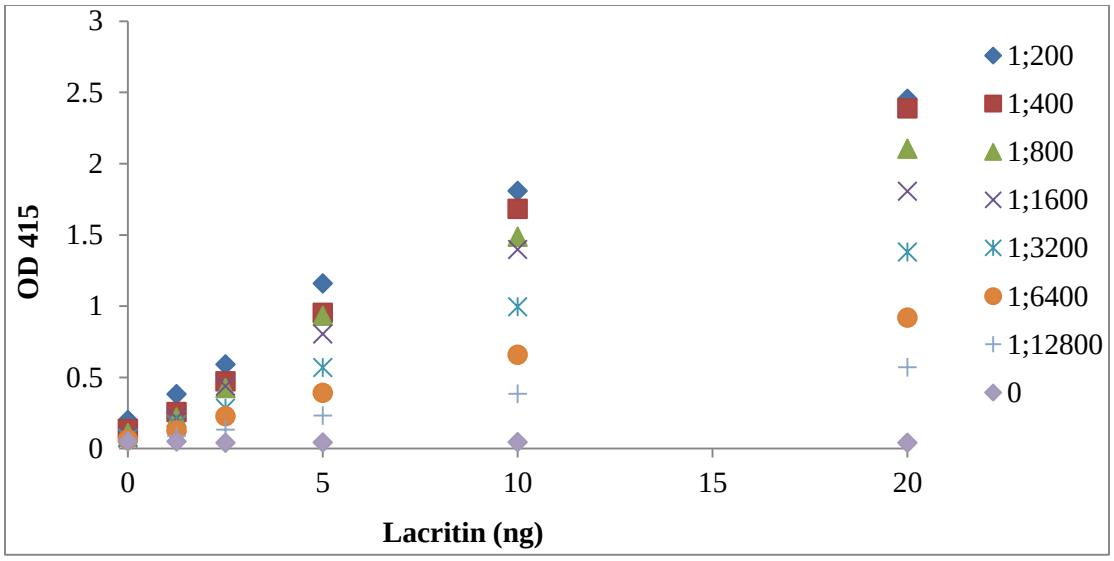


Figure 7. Checkerboard titration of 6924 antibodies. A checkerboard titration where lacritin was diluted from 1.5 to 20 ng/mL, the primary antibody was diluted to 1:400 and the secondary antibody was diluted from 1:200 to 1:12800.

Indirect ELISA Assay

An indirect ELISA was used to quantify lacritin in each tear sample. The assay was developed using 10 week Protein A purified anti-cLACRT NT antiserum from rabbit 6924 as the primary antibody and HRP-conjugated anti-rabbit secondary antibody. All standard curve R^2 values were above 0.96. Samples were run in triplicate with a standard curve done in duplicate plates. Due to technical limitations of the assay, 16 ng lacritin/50 ng total protein is the highest concentration that can be detected without extrapolation. Thus, samples that had concentrations higher than 16 ng lacritin are categorized as 16 ng or greater.

Lacritin concentrations in the 32 dry eye samples ranged from 0-16 ng /per 50 ng total tear protein (median 4.38 ng/50 ng total; average 5.80 ng/50 ng total). The range of lacritin in the normal samples was 2.32-16 ng lacritin per 50 ng total tear protein (median 14.83 ng/50 ng total; average 13.29 ng/50 ng total). Figure 8 shows a representative standard curve generated from indirect ELISA. Tables 3 and 4 show summaries of the lacritin concentrations in the dry eye and normal canines, respectively. Raw indirect ELISA data can be found in Appendix B.

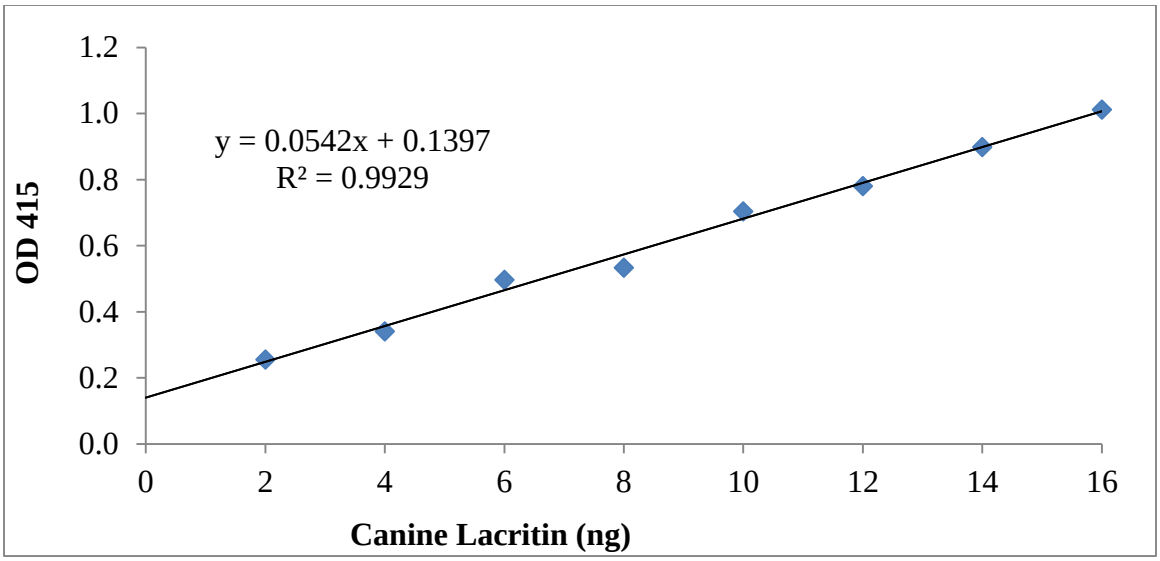


Figure 8. Standard Curve using 6924 antibodies. An example of a standard curve of canine lacritin concentrations used for indirect ELISA quantification.

Table 3. Summary of lacritin concentrations in dry eye canines The eye of origin (OD or OS) and concentration of each sample is indicated. Samples are listed in ascending order of average (left and right) lacritin concentration.

Lacritin Concentration in Dry Eye Canines

Sample ID	Eye	Lacritin (ng/ 50 ng total protein)	Sample ID	Eye	Lacritin (ng/ 50 ng total protein)
136361	OD	0.0	**134443**	OD	5.7
	OS	0.0		OS	4.6
106997	OD	0.0	**130826**	OD	9.0
	OS	0.0		OS	5.3
134918	OD	0.0	**134882**	OD	0.0
	OS	0.0		OS	16.0
137185	OS	0.0	**134668**	OD	15.9
102059	OS	0.0		OS	0.0
92935	OD	0.0	**135100**	OD	9.7
	OS	1.5	**124839**	OD	7.8
134564	OD	1.7		OS	13.5
114039	OD	6.5	**131139**	OS	13.9
	OS	0.0	**134989**	OD	15.0
122428	OD	7.4		OS	16.0
	OS	0.0	**93777**	OD	16.0
134581	OS	4.1		OS	16.0

Table 4. Summary of lacritin concentrations in normal canines. The eye of origin (OD or OS) and concentration of each sample is indicated. Samples are listed in ascending order of average (left and right) lacritin concentration.

Lacritin Concentration in Normal Canines

Sample ID	Eye	Lacritin (ng/ 50 ng total protein)	Sample ID	Eye	Lacritin (ng/ 50 ng total protein)
131139	OD	2.3	**87637**	OD	13.4
125091	OD	16.0		OS	16.0
	OS	3.7	**102059**	OD	14.8
117503	OD	4.2	**137477**	OD	16.0
	OS	16.0		OS	13.9
121747	OD	13.5	**135570**	OS	15.4
	OS	9.0	**119030**	OD	16.0
120039	OD	10.4		OS	16.0
	OS	12.3	**129754**	OD	16.0
101803	OD	10.7		OS	16.0
	OS	12.8	**114897**	OD	16.0
135303	OD	12.7		OS	16.0
	OS	11.8	**137481**	OD	16.0
135100	OS	14.2		OS	16.0
137478	OD	16.0	**128415**	OD	16.0
	OS	12.9		OS	16.0

Western Blots

A western blot was performed to visually detect lacritin concentration. Anti-cLACRT NT PA purified antiserum from rabbit 6924 was used as the primary antibody and HRP-conjugated antibody was used as the secondary antibody. Normal samples produced a prominent band at 18 kDa. This band was fainter, or absent, in dry eye samples. Western blots of all eight sets of samples are shown in Figure 9.

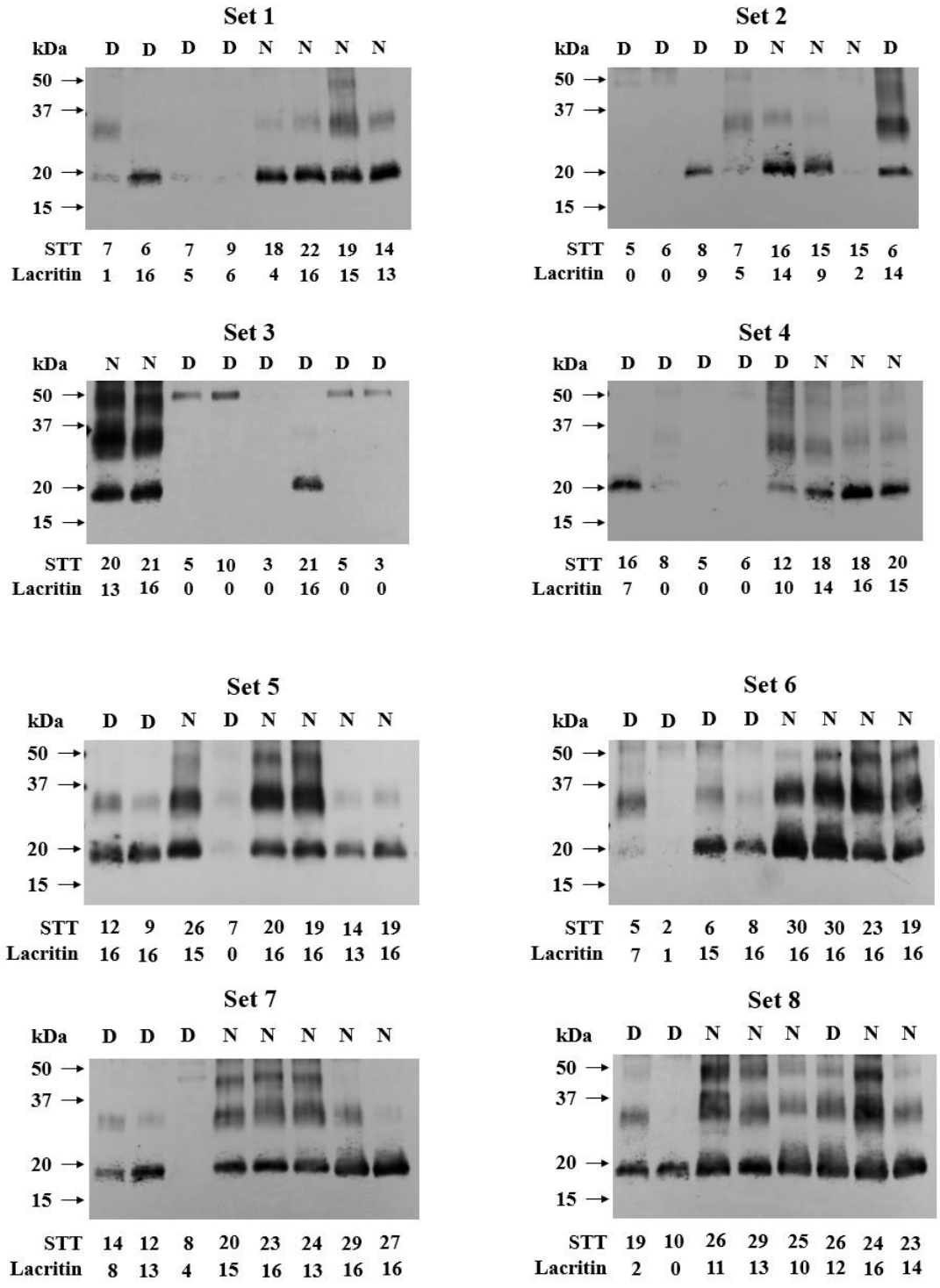


Figure 9. Western blot analysis of dry eye and normal samples. Western blot analysis of the samples with their Schirmer Tear Test (STT) values and lacritin concentrations, as determined by indirect ELISA. An STT value below 15 indicates dry eye, while values above indicate normal tearing levels. The band corresponding to monomeric canine lacritin is seen at ~18 kDa. Higher molecular

weight moeities are cross-linked variations of lacritin. D = Dry eye tear samples and N = Normal tear samples. Lacritin values are ng per 50 ng total tear protein as determined by the ELISA.

To better understand these western blot data, one must compare it to the Schirmer Tear Test (STT) values. A STT is a test used to measure tearing levels in both dogs and humans to help diagnose dry eye. For canines, any value under 15 indicates dry eye and any value above 15 indicates normal tearing. A scatter plot showing the correlation between total tear protein and STT values is shown is shown in Figure 10, and a scatter plot showing the correlation between lacritin concentration and STT values is shown in Figure 11.

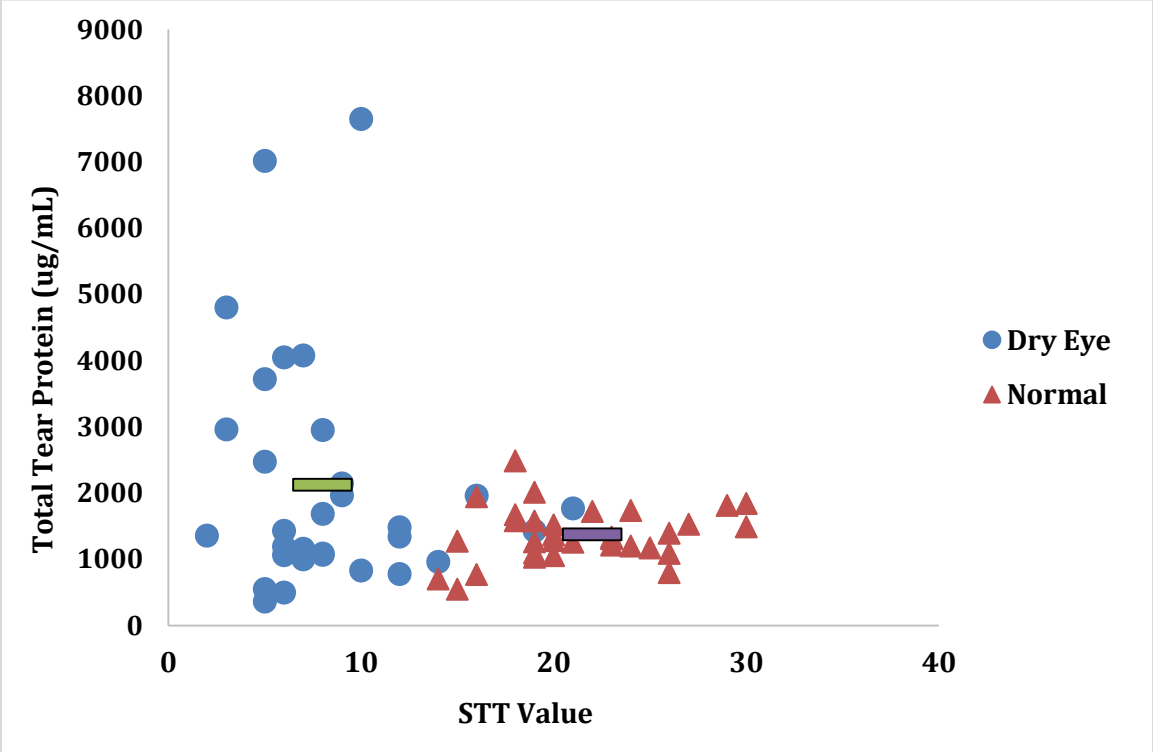


Figure 10. Dry eye and normal total tear protein as a function of STT values. A scatter plot showing the total tear protein of the normal and dry eye samples. An STT value is a measure of tearing in the eyes and a value below 15 indicates dry eye and a value above 15 indicates normal tearing levels. The bar in the middle of each cluster of points represents the average for that data set.

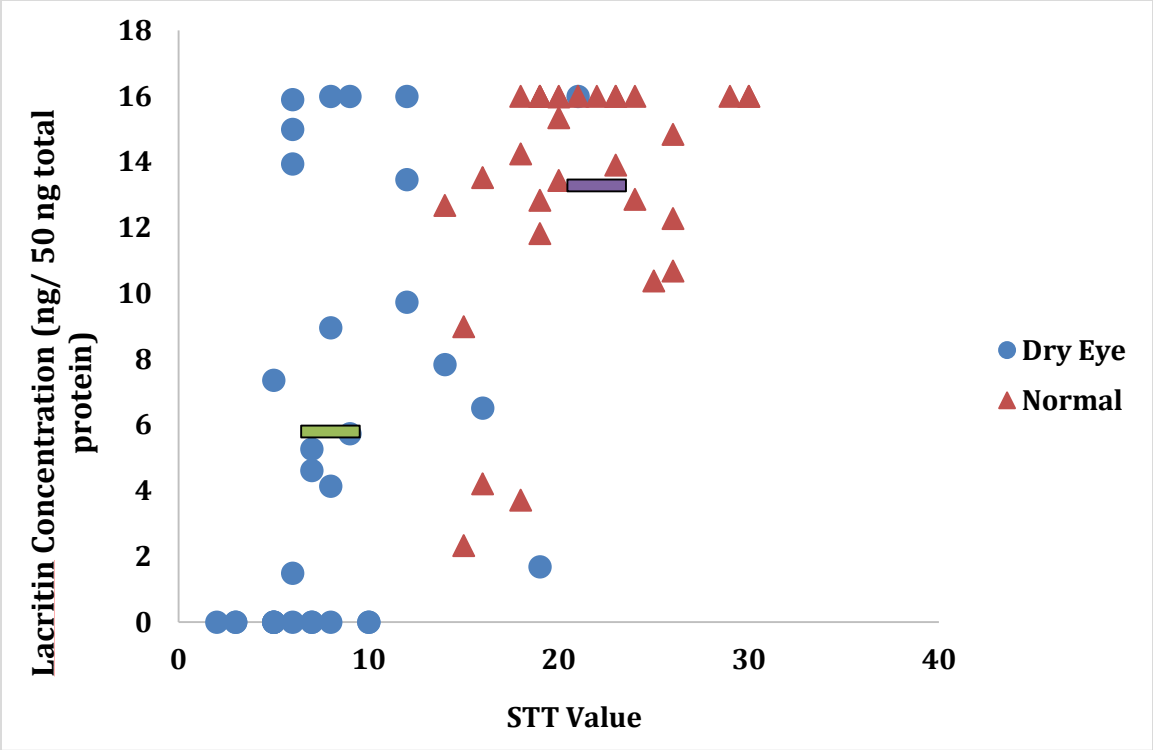


Figure 11. Dry eye and normal lacritin concentrations as a function of STT values. A scatter plot showing lacritin concentration as determined by the ELISA as a function of STT values in the dry eye and normal samples. An STT value is a measure of tearing in the eyes and a value below 15 indicates dry eye and a value above 15 indicates normal tearing levels. The bar in the middle of each cluster of points represents the average for that data set.

DISCUSSION

In this clinical study, 64 samples of canine tears, 32 dry eye and 32 normal, were collected at the VA-MD Regional College of Veterinary Medicine at Virginia Tech by Dr. Julie Disney. The samples were sent to JMU and assayed for total tear protein by BCA assay and lacritin concentration by indirect ELISA. Lacritin was visually detected in samples using a western blot.

According to this study, canines with dry eye had variable total tear protein levels, ranging from 367.1-7655 μg/mL with a standard deviation of 1737.5 μg/mL, while the normal tears had consistent total tear protein values between 550.6-2485 μg/mL with a standard deviation of 403.8 μg/mL (Figure 10). The three highest dry eye samples (7655, 7018 and 4807 μg/mL) were collected from canines that are non-responsive to clinical treatment for dry eye. It is possible that their treatment may have stimulated excessive tear protein production, explaining the atypically high concentrations. It is also possible that canines with dry eye produce more tear proteins in an attempt to compensate for their condition.

Lacritin concentrations were reduced in dry eye samples compared to normal samples (Figure 11). Dry eye samples had an average of 5.8 ng and a standard deviation of 4.3 ng lacritin per total ng protein while normal samples had an average of 13.3 ng and a standard deviation of 3.8 ng lacritin (Figure 11). Interesting to note, the three highest dry eye total tear protein samples mentioned above had undetectable levels of lacritin by ELISA. Thus although these animals high total concentrations of tear proteins, lacritin is absent, which may be correlated with their lack of response to treatments. Variability within the sample set can be expected in a clinical study incorporating many different breeds, ages, and genders of canines. It is also important to note that samples were collected from canines during diagnosis or treatment of dry eye. Such variability in clinical progression can also explain variation within the data set.

Another point of interest is that some of the western blots detect lacritin complexes greater than 18 kDa. This indicates that the anti-canine LAC antibody is detecting a cross-linked lacritin. It has been shown that tissue transglutaminase cross-links (human?) lacritin and forms higher molecular weight complexes that appear above ~18 kD on western blots (Velez, 2013). Cross-linked lacritin has been shown to be inactive (Velez, 2013). Our ELISA does not detect the cross-linked lacritin, as some of the western blot samples with high

molecular weight forms of lacritin have undetectable concentrations of lacritin by ELISA. This is likely due to the denaturing step of the western blot, which is not performed in the ELISA. It is possible that upon crosslinking, the epitope detected by the antibodies must be buried and inaccessible. Additionally, two samples on the Set 2 western blot appear to be switched; the normal sample has a lacritin concentration of 2 ng and has the absence of the band where lacritin should be on the blot, while the dry eye sample has lacritin concentration of 14 ng and has a visible band where lacritin should be. It is possible that these samples accidently got switched or mislabeled during collection, or during the processing done at JMU.

In conclusion, a system for detecting canine lacritin in canine tear samples was developed and used to quantify and visually detect lacritin concentrations. Dye eye samples had significantly lower concentrations of lacritin compared to normal samples, reinforcing the hypothesis that lacritin replacement may be an effective therapeutic for dry eye.

Future work to continue this project include both canine and human clinical trials, which are both needed to bring a drug to market. As for a canine therapeutic, the next step would be to conduct a study in which lacritin is topically applied to the eyes of healthy and dry eye dogs to see if it improves basal tearing levels. The ELISA which was developed in the study can be used to identify canines with low lacritin levels to enroll in the canine clinical studies. This ELSIA can also be used to monitor lacritin levels in the treated canines over time to assess the effectiveness of lacritin replacement therapy. Successful treatment of canine dry eye with lacritin holds promising results for treating human dry eye. Human clinical trials are needed to confirm the efficacy and safety of lacritin as a therapeutic in humans, and once passed, lacritin can be brought to market as a human therapeutic.

BIBLIOGRAPHY

Enghauser, A. (2015). *Development of an immunodiagnostic assay for canine tear lacritin.* Unpublished B.S, James Madison University, USA.

"Keratoconiuntivis Sicca". The Merck Manual, Home Edition. Merck & Co., Inc. 2003-02-01.

Koo, B. S., Lee, D. Y., Ha, H. S., Kim, J. C., & Kim, C. W. (1006). *Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis* Retrieved from 2005 May-Jun database.

Laurie, D. E., Splan, R. K., Green, K., Still, K. M., McKown, R. L., & Laurie, G. W. (2012). Detection of prosecretory mitogen lacritin in nonprimate tears primarily as a C-terminal-like fragment. *Investigative Ophthalmology & Visual Science, 53*(10), 6130-6136. doi:10.1167/iovs.11-8567

Ma, P., Wang, N., McKown, R. L., Raab, R. W., & Laurie, G. W. (2007). Focus on molecules: Lacritin. *Experimental Eye Research, 86*(3), 457-458. doi:10.1016/j.exer.2007.01.025

McKown, R. L., Coleman Frazier, E. V., Zadrozny, K. K., Deleault, A. M., Raab, R. W., Ryan, D. S., et al. (0219). *A cleavage-potentiated fragment of tear lacritin is bactericidal* [Bacterial Metabolism; Cornea; Eye; Innate Immunity; Lacritin; Phosphatidylethanolamine; Protein Targeting; Serine Protease; Tears EDAT- 2014/06/20 06:00 MHDA- 2015/02/20 06:00 CRDT- 2014/06/20 06:00 PHST- 2014/06/18 [aheadofprint] AID - M114.570143 [pii] AID - 10.1074/jbc.M114.570143 [doi] PST - ppublish] Retrieved from 2014 Aug 8 database.

McKown, R.L., Tate, A.C., Enghauser, A.M., Soyars C.L., Raab, R.W., Laurie, G.W., Herring, I.P.. (2015). Canine Tear lacritin is Down Regulated in Clinical Dry Eye. ARVO Poster Presentation, May 3, 2015.

Ofri, R., Lambrou, G. N., Allgoewer, I., Graenitz, U., Pena, T.M., Spiess, B. M., Latour, E., et al. (2009). *Clinical evaluation of pimecrolimus eye drops for treatment of canine*

keratoconjunctivitis sicca: A comparison with cyclosporine A Retrieved from 2009 Jan database.

Samudre, S., Lattanzio FA Jr FAU - Lossen,,Victoria, Lossen, V. F., Hosseini A FAU - Sheppard,,John D.,Jr, Sheppard JD Jr FAU - McKown, Robert,L., FAU, M. R., et al. (1005). *Lacritin, a novel human tear glycoprotein, promotes sustained basal tearing and is well tolerated* Retrieved from 2011 Aug database.

Sanghi, S., Kumar, R. F., Lumsden, A. F., Dickinson, D. F., Klepeis, V. F., Trinkaus-Randall V FAU - Frierson,,H.F.,Jr, et al. (0809). *cDNA and genomic cloning of lacritin, a novel secretion enhancing factor from the human lacrimal gland* Retrieved from 2001 Jun 29 database.

Seifert, K., Gandia, N. C., Wilburn, J. K., Bower, K. S., Sia, R. K., Ryan, D. S., et al. (2012). Tear lacritin levels by age, sex, and time of day in healthy adults. *Investigative Ophthalmology & Visual Science, 53*(10), 6610-6616. doi:10.1167/iovs.11-8729

Tate, A. (2014). *Canine clinical study for tear lacritin as a treatment for dry eye.* Unpublished B.S., James Madison University, USA.

Tate, A.C., Soyars, C.L., Raab, R.W., Herring, I.P., McKown, R.L.. (2013). Canine Clinical Study for Tear Lacritin as a Treatment for Dry Eye. 4-VA Poster Presentation, September 26, 2013.

Tsai, P. S., Evans, J. E., Green, K. M., Sullivan, R. M., Schaumberg, S. M., Dana, R. R., et al. (0328). *Proteomic analysis of human meibomian gland secretions* Retrieved from 2006 Mar database.

Velez, V. F., Romano, J.A.,McKown, R.L., Green, K. F., Zhang L., Raab, R,W., et al. (0514). *Tissue transglutaminase is a negative regulator of monomeric lacritin bioactivity* Retrieved from 2013 Mar database.

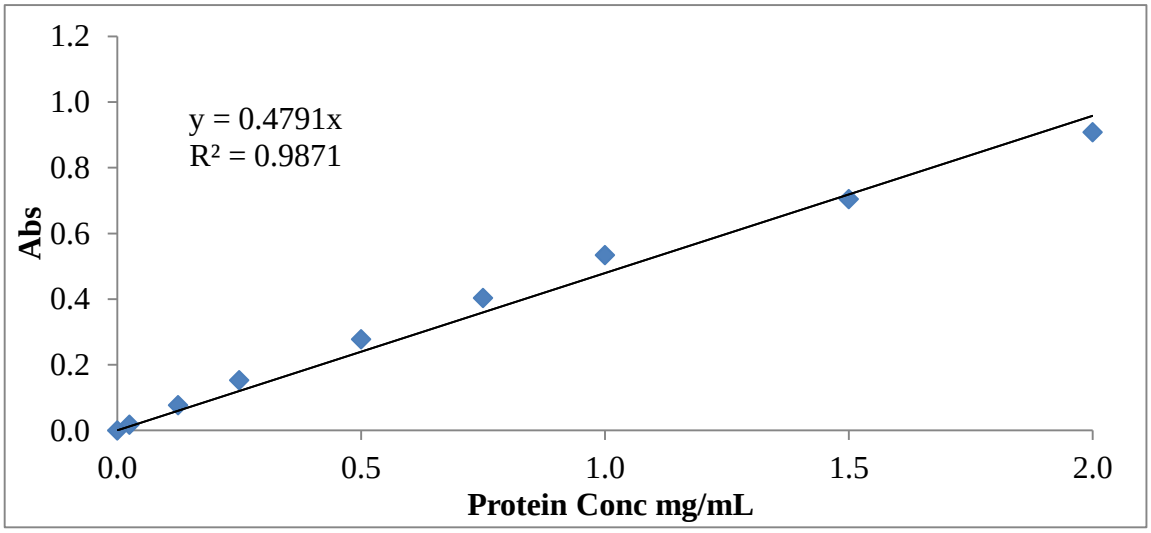
Williams, D. L. (0512). *Immunopathogenesis of keratoconjunctivitis sicca in the dog* Retrieved from 2008 Mar database.

APPENDIX A

Raw data from the BCA assay is shown below. A darker shade of orange indicates a higher standard deviation of the absorbances. Mathematically, the corrected column represents the average absorbance with the background absorbance subtracted out.

BCA Canine Samples 2-17-16 Set 1

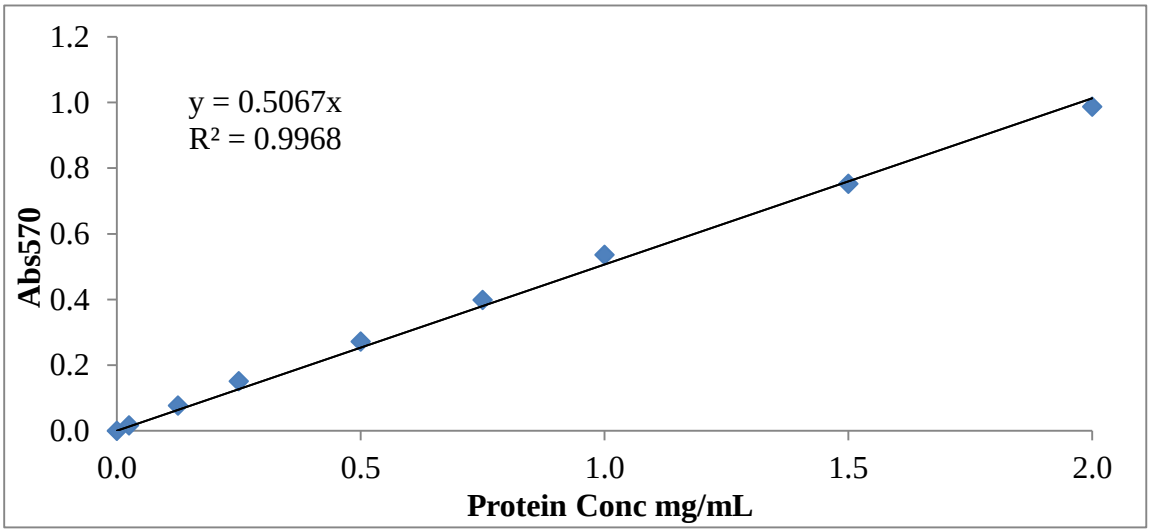
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
0.986	0.983	0.985	0.002	2.000	0.909
0.804	0.757	0.781	0.033	1.500	0.705
0.653	0.567	0.610	0.061	1.000	0.535
0.484	0.475	0.480	0.006	0.750	0.404
0.360	0.348	0.354	0.008	0.500	0.279
0.229	0.228	0.229	0.001	0.250	0.153
0.156	0.150	0.153	0.004	0.125	0.078
0.092	0.095	0.094	0.002	0.025	0.018
0.074	0.077	0.076	0.002	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OS 68	0.153	0.158	0.156	0.004	0.080	0.16698	166.98	1002
OD 68	0.171	0.171	0.171	0.000	0.096	0.19933	199.33	1196
OS 43	0.178	0.159	0.169	0.013	0.093	0.19411	194.11	1165
OD 43	0.251	0.243	0.247	0.006	0.172	0.35796	357.96	2148
OS 91	0.185	0.219	0.202	0.024	0.127	0.26404	264.04	1584
OD 91	0.229	0.197	0.213	0.023	0.138	0.28700	287	1722
OS 03	0.172	0.181	0.177	0.006	0.101	0.21081	210.81	1265
OD 03	0.222	0.240	0.231	0.013	0.156	0.32457	324.57	1947

BCA Canine Samples 2-17-16 Set 2

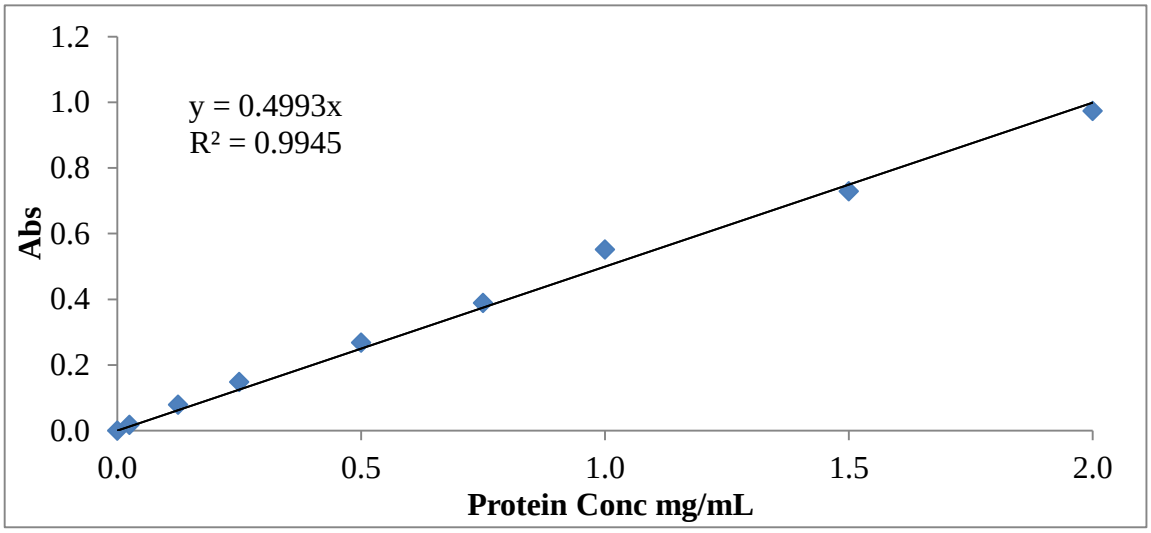
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
1.073	1.053	1.063	0.014	2.000	0.988
0.839	0.817	0.828	0.016	1.500	0.753
0.643	0.579	0.611	0.045	1.000	0.536
0.479	0.469	0.474	0.007	0.750	0.399
0.351	0.344	0.348	0.005	0.500	0.273
0.223	0.230	0.227	0.005	0.250	0.152
0.153	0.152	0.153	0.001	0.125	0.078
0.091	0.093	0.092	0.001	0.025	0.017
0.074	0.076	0.075	0.001	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 35	0.105	0.107	0.106	0.001	0.031	0.06118	61.18	367.1
OS 35	0.117	0.118	0.118	0.001	0.043	0.08388	83.876	503.3
OD 26	0.170	0.164	0.167	0.004	0.092	0.18157	181.57	1089
OS 26	0.172	0.170	0.171	0.001	0.096	0.18946	189.46	1137
OD 47	0.168	0.151	0.160	0.012	0.085	0.16677	166.77	1001
OS 47	0.153	0.141	0.147	0.008	0.072	0.14210	142.1	852.6
OD 139	0.183	0.182	0.183	0.001	0.108	0.21216	212.16	1273
OS 139	0.198	0.194	0.196	0.003	0.121	0.23880	238.8	1433

BCA Canine Samples 2-22-16 Set 3

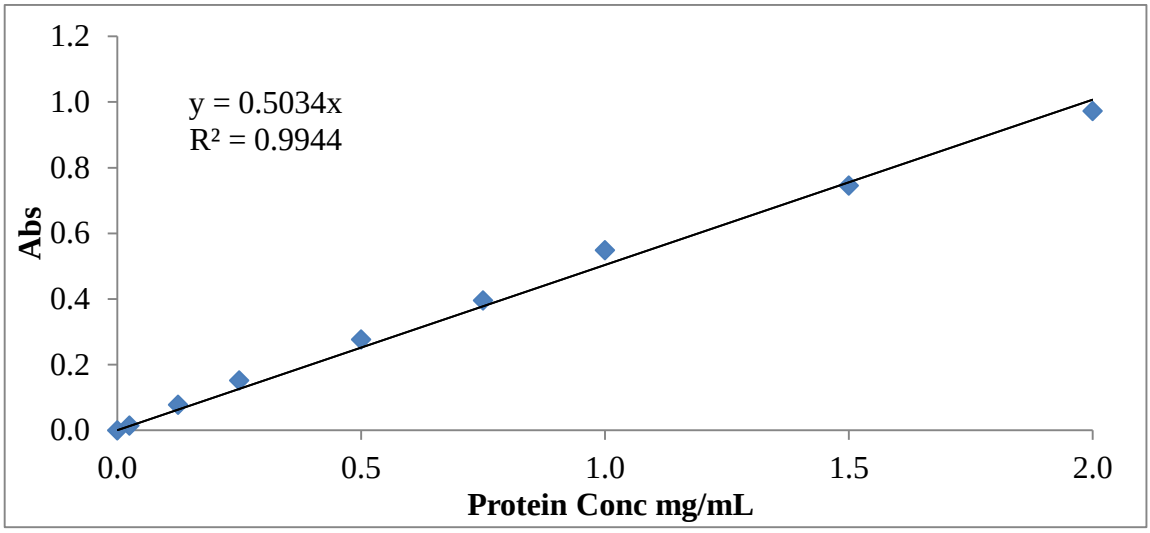
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
1.053	1.049	1.051	0.003	2.000	0.974
0.823	0.788	0.806	0.025	1.500	0.729
0.674	0.583	0.629	0.064	1.000	0.552
0.474	0.457	0.466	0.012	0.750	0.389
0.350	0.339	0.345	0.008	0.500	0.268
0.228	0.222	0.225	0.004	0.250	0.148
0.154	0.158	0.156	0.003	0.125	0.079
0.094	0.094	0.094	0.000	0.025	0.017
0.076	0.078	0.077	0.001	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 37	0.203	0.204	0.204	0.001	0.127	0.25335	253.35	1520
OS 37	0.182	0.182	0.182	0.000	0.105	0.21029	210.29	1262
OD 97	0.690	0.632	0.661	0.041	0.584	1.16964	1169.6	7018
OS 97	0.760	0.668	0.714	0.065	0.637	1.27579	1275.8	7655
OD 82	0.505	0.449	0.477	0.040	0.400	0.80112	801.12	4807
OS 82	0.233	0.216	0.225	0.012	0.148	0.29541	295.41	1772
OD 61	0.329	0.445	0.387	0.082	0.310	0.62087	620.87	3725
OS 61	0.350	0.298	0.324	0.037	0.247	0.49469	494.69	2968

BCA Canine Samples 2-22-16 Set 4

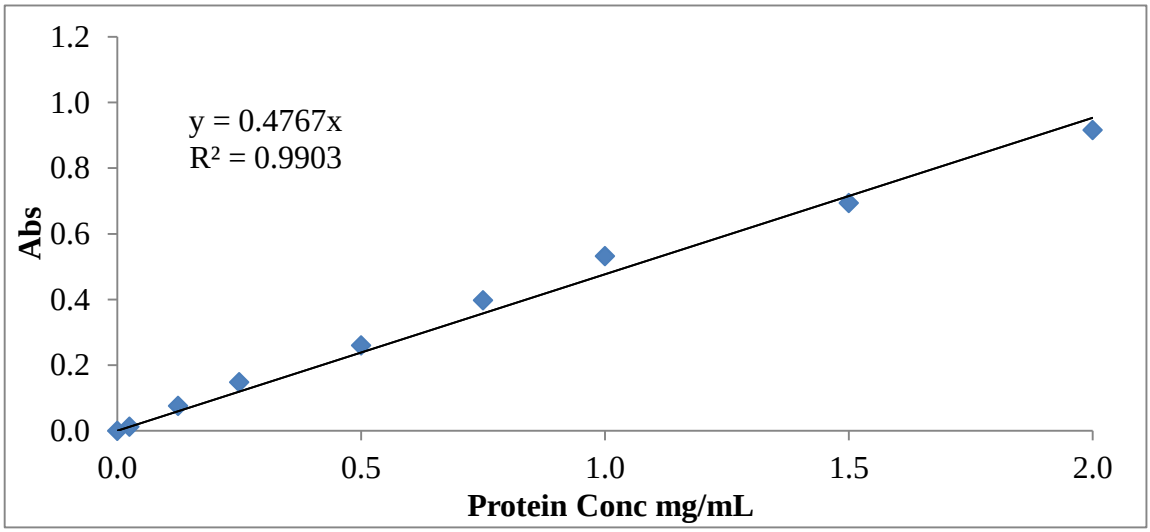
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
1.043	1.058	1.051	0.011	2.000	0.973
0.848	0.799	0.824	0.035	1.500	0.746
0.683	0.570	0.627	0.080	1.000	0.549
0.483	0.465	0.474	0.013	0.750	0.397
0.362	0.347	0.355	0.011	0.500	0.277
0.235	0.224	0.230	0.008	0.250	0.152
0.156	0.156	0.156	0.000	0.125	0.079
0.093	0.093	0.093	0.000	0.025	0.016
0.076	0.079	0.078	0.002	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 039	0.240	0.245	0.243	0.004	0.165	0.32777	327.77	1967
OS 039	0.171	0.164	0.168	0.005	0.090	0.17878	178.78	1073
OD 18	0.363	0.208	0.286	0.110	0.208	0.41319	413.19	2479
OS 18	0.174	0.160	0.167	0.010	0.090	0.17779	177.79	1067
OD 100	0.192	0.189	0.191	0.002	0.113	0.22447	224.47	1347
OS 100	0.304	0.268	0.286	0.025	0.209	0.41418	414.18	2485
OD 30	0.214	0.222	0.218	0.006	0.141	0.27910	279.1	1675
OS 30	0.190	0.195	0.193	0.004	0.115	0.22845	228.45	1371

BCA Canine Samples 2-24-16 SET 5

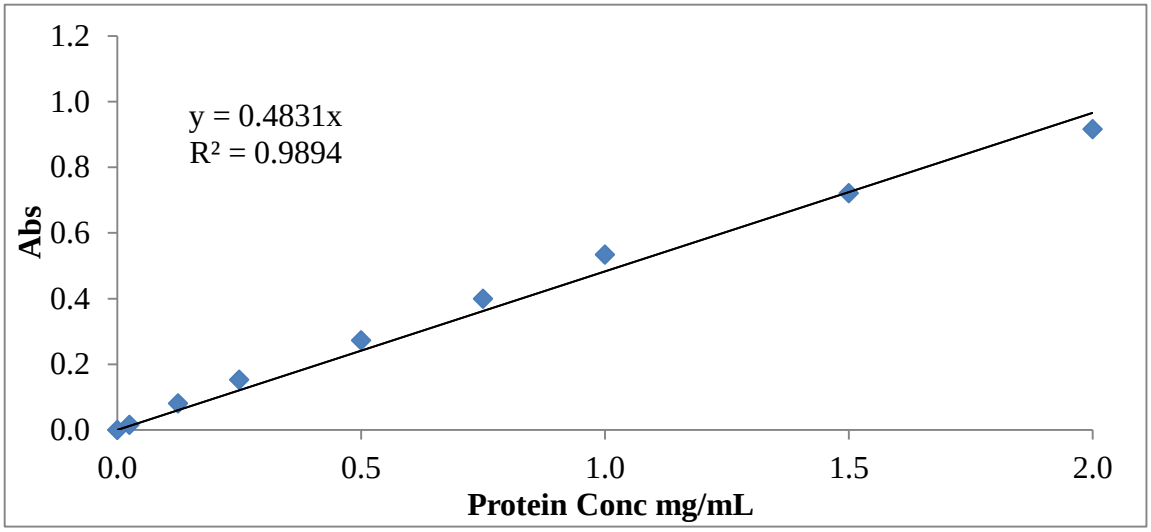
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
0.995	0.993	0.994	0.001	2.000	0.916
0.783	0.762	0.773	0.015	1.500	0.695
0.672	0.548	0.610	0.088	1.000	0.532
0.463	0.488	0.476	0.018	0.750	0.398
0.351	0.325	0.338	0.018	0.500	0.260
0.225	0.227	0.226	0.001	0.250	0.148
0.153	0.156	0.155	0.002	0.125	0.077
0.092	0.090	0.091	0.001	0.025	0.013
0.077	0.079	0.078	0.001	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 777	0.202	0.190	0.196	0.008	0.118	0.24754	247.54	1485
OS 777	0.240	0.229	0.235	0.008	0.157	0.32830	328.3	1970
OD 059	0.146	0.137	0.142	0.006	0.064	0.13321	133.21	799.2
OS 059	0.548	0.257	0.403	0.206	0.325	0.68072	680.72	4084
OD 754	0.179	0.181	0.180	0.001	0.102	0.21397	213.97	1284
OS 754	0.167	0.163	0.165	0.003	0.087	0.18250	182.5	1095
OD 303	0.141	0.127	0.134	0.010	0.056	0.11747	117.47	704.8
OS 303	0.190	0.130	0.160	0.042	0.082	0.17202	172.02	1032

BCA Canine Samples 2-24-16 SET 6

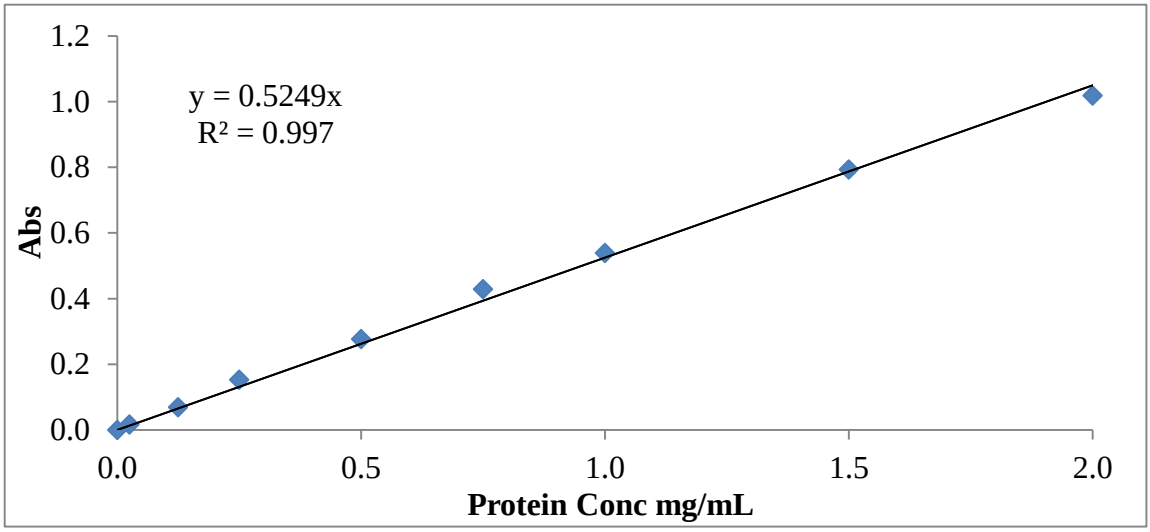
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
0.973	0.968	0.971	0.004	2.000	0.916
0.782	0.769	0.776	0.009	1.500	0.721
0.656	0.521	0.589	0.095	1.000	0.534
0.456	0.453	0.455	0.002	0.750	0.400
0.330	0.325	0.328	0.004	0.500	0.273
0.207	0.208	0.208	0.001	0.250	0.153
0.137	0.133	0.135	0.003	0.125	0.081
0.070	0.070	0.070	0.000	0.025	0.016
0.054	0.055	0.055	0.001	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 428	0.099	0.099	0.099	0.000	0.045	0.09211	92.113	552.7
OS 428	0.150	0.178	0.164	0.020	0.110	0.22666	226.66	1360
OD 989	0.378	0.384	0.381	0.004	0.327	0.67584	675.84	4055
OS 989	0.271	0.314	0.293	0.030	0.238	0.49265	492.65	2956
OD 897	0.209	0.197	0.203	0.008	0.149	0.30739	307.39	1844
OS 897	0.174	0.175	0.175	0.001	0.120	0.24840	248.4	1490
OD 481	0.161	0.163	0.162	0.001	0.108	0.22252	222.52	1335
OS 481	0.215	0.148	0.182	0.047	0.127	0.26289	262.89	1577

BCA Canine Samples 2-26-16 SET 7

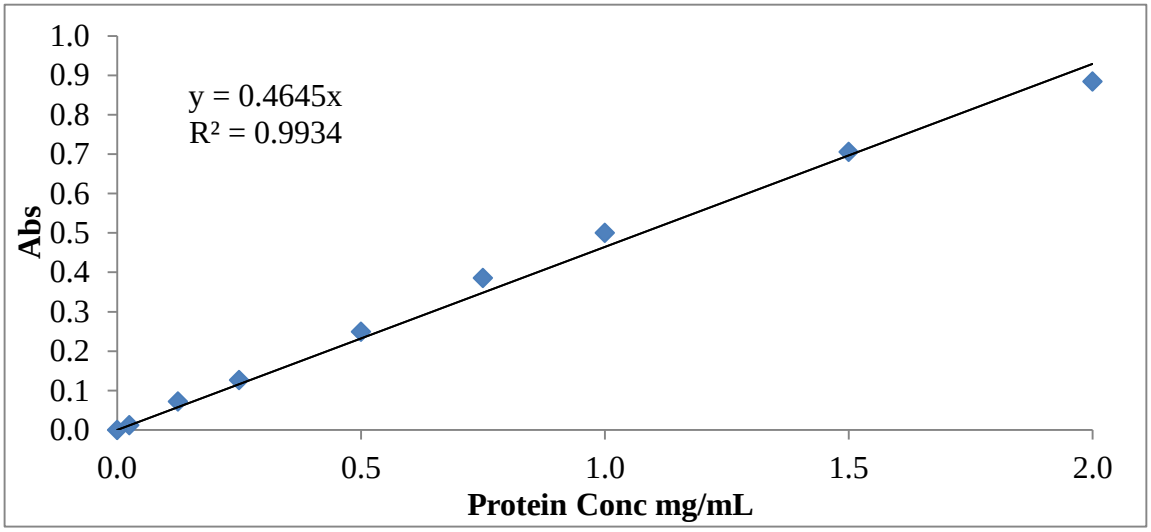
ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
1.120	1.095	1.108	0.018	2.000	1.019
0.897	0.868	0.883	0.021	1.500	0.794
0.671	0.585	0.628	0.061	1.000	0.539
0.525	0.511	0.518	0.010	0.750	0.429
0.370	0.361	0.366	0.006	0.500	0.277
0.241	0.243	0.242	0.001	0.250	0.153
0.159	0.158	0.159	0.001	0.125	0.070
0.105	0.107	0.106	0.001	0.025	0.017
0.088	0.090	0.089	0.001	0.000	0.000



Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 839	0.187	0.160	0.174	0.019	0.085	0.16098	160.98	965.9
OS 839	0.157	0.158	0.158	0.001	0.069	0.13050	130.5	783
OS 581	0.241	0.233	0.237	0.006	0.148	0.28196	281.96	1692
OS 570	0.181	0.182	0.182	0.001	0.093	0.17622	176.22	1057
OD 478	0.194	0.197	0.196	0.002	0.107	0.20290	202.9	1217
OS 478	0.274	0.208	0.241	0.047	0.152	0.28958	289.58	1737
OD 415	0.246	0.250	0.248	0.003	0.159	0.30291	302.91	1817
OS 415	0.221	0.225	0.223	0.003	0.134	0.25529	255.29	1532

BCA Canine Samples 2-26-16 SET 8

ABS 1	ABS 2	AVG	ST. DEV.	Conc (mg/mL)	Corrected
0.944	0.982	0.963	0.027	2.000	0.885
0.802	0.766	0.784	0.025	1.500	0.706
0.631	0.526	0.579	0.074	1.000	0.500
0.480	0.448	0.464	0.023	0.750	0.386
0.321	0.334	0.328	0.009	0.500	0.249
0.208	0.202	0.205	0.004	0.250	0.127
0.152	0.150	0.151	0.001	0.125	0.073
0.089	0.092	0.091	0.002	0.025	0.012
0.076	0.081	0.079	0.004	0.000	0.000



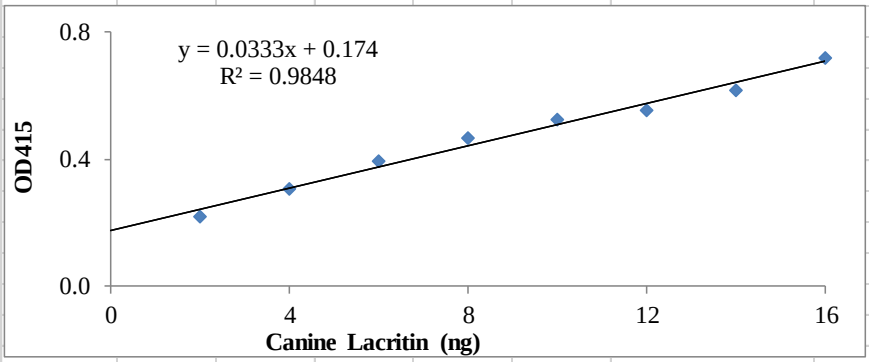
Sample	ABS 1	ABS 2	AVG	ST DEV	corrected	mg/mL	ug/mL	6x
OD 564	0.215	0.164	0.190	0.036	0.111	0.23897	238.97	1434
OS 185	0.145	0.141	0.143	0.003	0.065	0.13886	138.86	833.2
OD 803	0.186	0.187	0.187	0.001	0.108	0.23251	232.51	1395
OS 803	0.243	0.226	0.235	0.012	0.156	0.33584	335.84	2015
OD 0039	0.180	0.159	0.170	0.015	0.091	0.19591	195.91	1175
OS 0039	0.176	0.150	0.163	0.018	0.085	0.18192	181.92	1091
OD 477	0.166	0.177	0.172	0.008	0.093	0.20022	200.22	1201
OS 477	0.184	0.176	0.180	0.006	0.102	0.21851	218.51	1311

APPENDIX B

Raw data from the ELISA is shown below. Darker shades of orange indicate a higher standard deviation in the three absorbances measured from each sample.

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

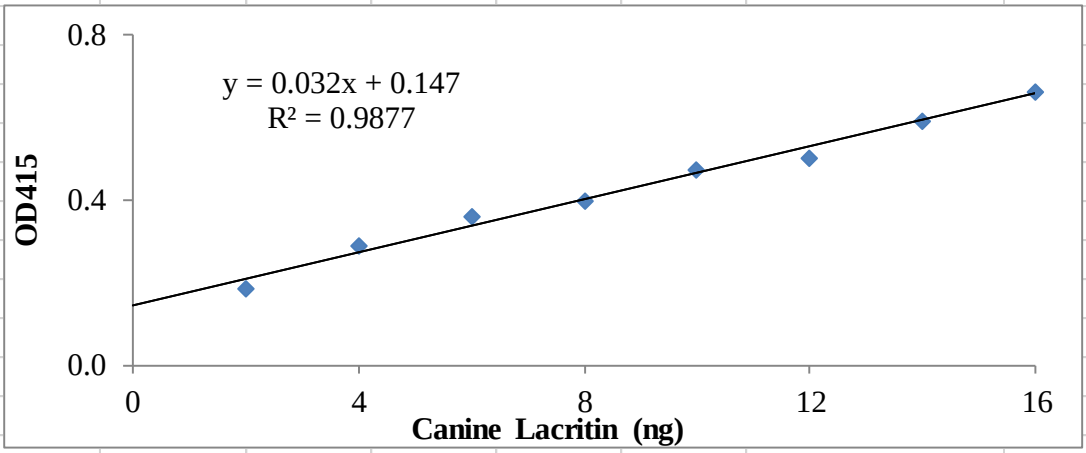
ng cLac	abs 1	abs 2	abs 3	average
16	0.735	0.719	0.700	0.718
14	0.596	0.622	0.637	0.618
12	0.542	0.524	0.588	0.551
10	0.513	0.528	0.536	0.526
8	0.460	0.469	0.462	0.464
6	0.400	0.394	0.384	0.393
4	0.300	0.312	0.307	0.306
2	0.217	0.214	0.221	0.217
0	0.136	0.062	0.092	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?
OS 68	0.199	0.187	0.203	0.203	0.008	0.901	DE
OD 68	0.697	0.728	0.733	0.733	0.020	16.769	DE
OS 43	0.313	0.327	0.333	0.333	0.010	4.793	DE
OD 43	0.361	0.375	0.362	0.362	0.008	5.662	DE
OS 91	0.293	0.307	0.290	0.290	0.009	3.506	N
OD 91	0.774	0.795	0.748	0.748	0.024	17.219	N
OS 03	0.846	0.821	0.825	0.825	0.013	19.524	N
OD 03	0.327	0.329	0.315	0.315	0.008	4.254	N

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

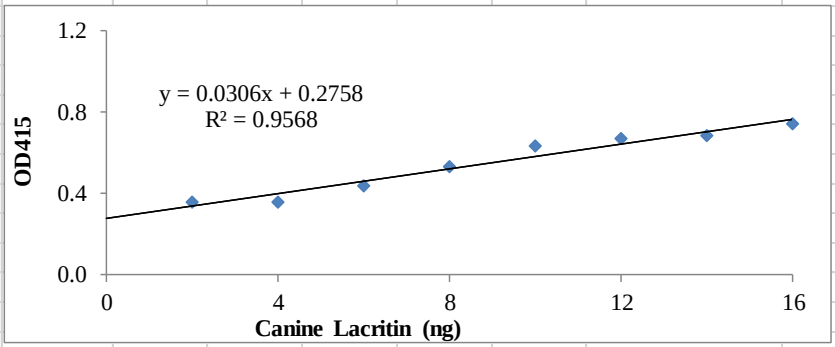
ng cLac	abs 1	abs 2	abs 3	average
16	0.647	0.673	0.677	0.666
14	0.581	0.596	0.600	0.592
12	0.491	0.507	0.517	0.505
10	0.473	0.486	0.462	0.474
8	0.382	0.395	0.427	0.401
6	0.352	0.368	0.369	0.363
4	0.275	0.294	0.298	0.289
2	0.181	0.189	0.190	0.187
0	0.048	0.049	0.050	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?
OS 68	0.167	0.191	0.162	0.173	0.016	0.823	DE
OD 68	0.652	0.585	0.647	0.628	0.037	15.031	DE
OS 43	0.293	0.279	0.296	0.289	0.009	4.448	DE
OD 43	0.334	0.336	0.329	0.333	0.004	5.813	DE
OS 91	0.274	0.262	0.281	0.272	0.010	3.917	N
OD 91	0.706	0.607	0.650	0.654	0.050	15.854	N
OS 03	0.785	0.772	0.782	0.780	0.007	19.771	N
OD 03	0.259	0.284	0.297	0.280	0.019	4.156	N

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

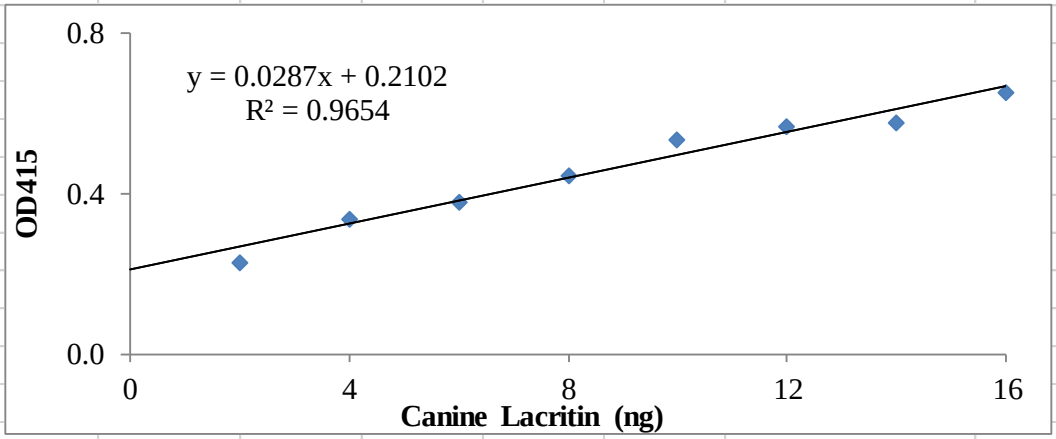
ng cLac	abs 1	abs 2	abs 3	average
16	0.736	0.696	0.801	0.744
14	0.730	0.657	0.659	0.682
12	0.658	0.651	0.690	0.666
10	0.603	0.668	0.639	0.637
8	0.510	0.542	0.540	0.531
6	0.413	0.459	0.436	0.436
4	0.354	0.363	0.357	0.358
2	0.389	0.395	0.282	0.355
0	0.051	0.056	0.052	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?	On treatment?
OD 35	0.356	0.359	0.367	0.361	0.006	2.773	DE	Yes
OS 35	0.419	0.446	0.470	0.445	0.026	5.529	DE	Yes
OD 26	0.728	0.731	0.717	0.725	0.007	14.691	DE	Yes
OS 26	0.563	0.517	0.574	0.551	0.030	9.004	DE	Yes
OD 47	0.118	0.117	0.132	0.122	0.008	-5.015	N	
OS 47	0.902	0.706	0.935	0.848	0.124	18.688	N	
OD 139	0.694	0.725	0.715	0.711	0.016	14.233	N	
OS 139	0.757	0.725	0.691	0.724	0.033	14.658	DE	Yes

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

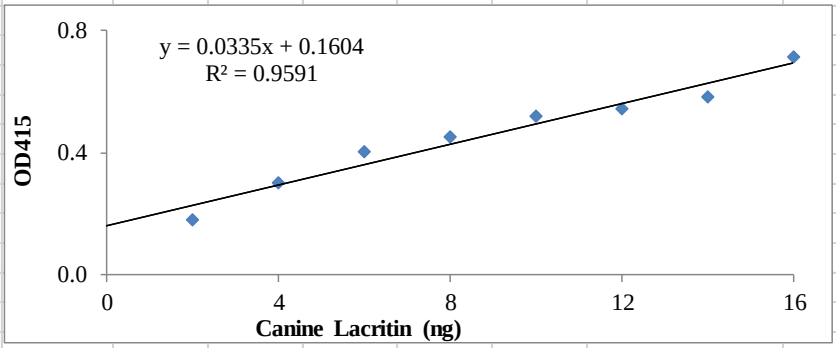
ng cLac	abs 1	abs 2	abs 3	average
16	0.647	0.650	0.673	0.657
14	0.551	0.575	0.606	0.577
12	0.552	0.554	0.607	0.571
10	0.532	0.530	0.555	0.539
8	0.427	0.436	0.477	0.447
6	0.359	0.392	0.398	0.383
4	0.330	0.342	0.349	0.340
2	0.217	0.239	0.239	0.232
0	0.048	0.049	0.054	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng
OD 35	0.299	0.279	0.287	0.288	0.010	3.717
OS 35	0.437	0.419	0.404	0.420	0.017	9.981
OD 26	0.672	0.701	0.691	0.688	0.015	22.731
OS 26	0.540	0.519	0.511	0.523	0.015	14.897
OD 47	0.100	0.097	0.117	0.105	0.011	-5.021
OS 47	0.821	0.857	0.815	0.831	0.023	29.534
OD 139	0.692	0.606	0.607	0.635	0.049	20.209
OS 139	0.680	0.683	0.696	0.686	0.009	22.651

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

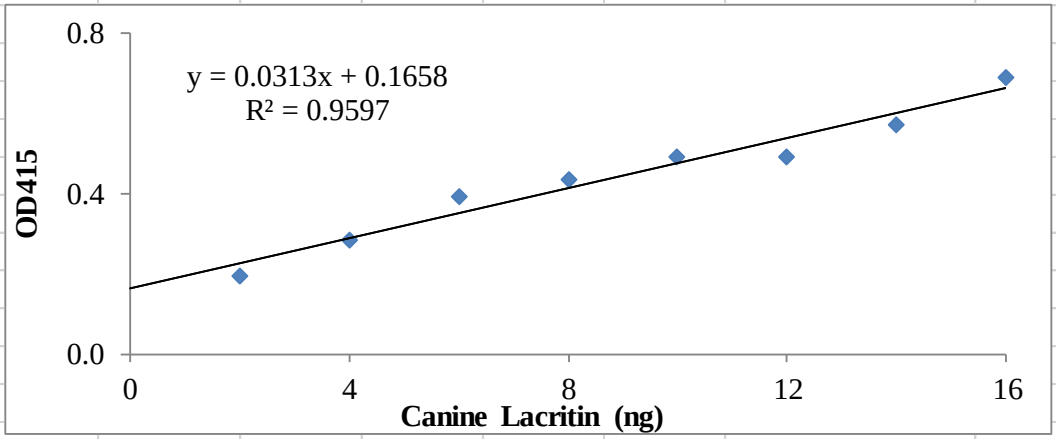
ng cLac	abs 1	abs 2	abs 3	average
16	0.685	0.743	0.714	0.714
14	0.591	0.619	0.532	0.581
12	0.538	0.539	0.550	0.542
10	0.512	0.514	0.537	0.521
8	0.449	0.458	0.450	0.452
6	0.386	0.402	0.413	0.400
4	0.284	0.304	0.316	0.301
2	0.183	0.165	0.195	0.181
0	0.068	0.054	0.057	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 37	0.642	0.614	0.618	0.625	0.015	13.859	N	
OS 37	0.903	0.881	0.912	0.899	0.016	22.038	N	
OD 97	0.093	0.072	0.088	0.084	0.011	-2.271	DE	Yes
OS 97	0.065	0.062	0.065	0.064	0.002	-2.878	DE	Yes
OD 82	0.126	0.111	0.097	0.111	0.015	-1.465	DE	Yes
OS 82	0.894	0.908	0.835	0.879	0.039	21.451	DE	Yes
OD 61	0.058	0.067	0.062	0.062	0.005	-2.927	DE	No
OS 61	0.193	0.167	0.144	0.168	0.025	0.227	DE	No

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

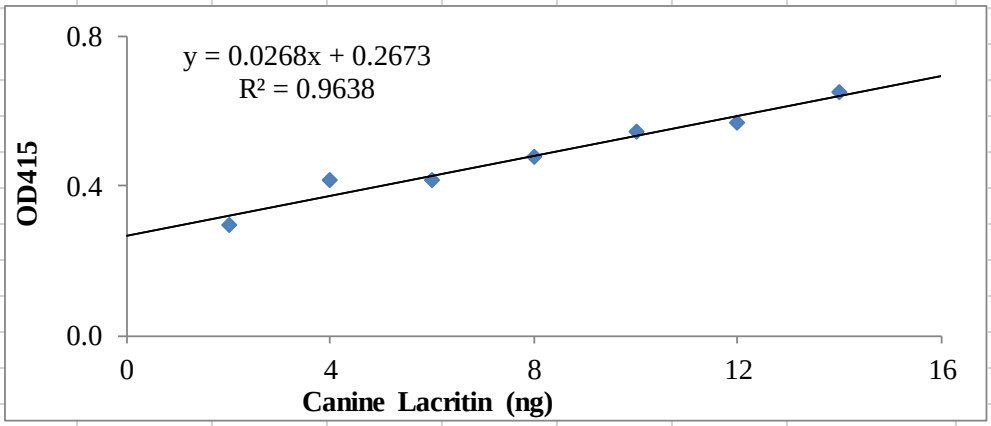
ng cLac	abs 1	abs 2	abs 3	average
16	0.693	0.697	0.688	0.693
14	0.570	0.595	0.560	0.575
12	0.496	0.500	0.492	0.496
10	0.496	0.495	0.491	0.494
8	0.438		0.436	0.437
6	0.403	0.391	0.392	0.395
4	0.283	0.284	0.299	0.289
2	0.191	0.197	0.207	0.198
0	0.074	0.075	0.061	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?
OD 37	0.558	0.581	0.599	0.579	0.021	13.014	N
OS 37	0.859	0.911	0.833	0.868	0.040	22.376	N
OD 97	0.100	0.090	0.112	0.101	0.011	-2.527	DE
OS 97	0.074	0.086	0.083	0.081	0.006	-3.166	DE
OD 82	0.107	0.123	0.113	0.114	0.008	-2.083	DE
OS 82	0.922	0.620	0.916	0.819	0.173	20.806	DE
OD 61	0.082	0.082	0.096	0.087	0.008	-2.982	DE
OS 61	0.163	0.147	0.195	0.168	0.024	-0.330	DE

Standard Curve 2-11-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

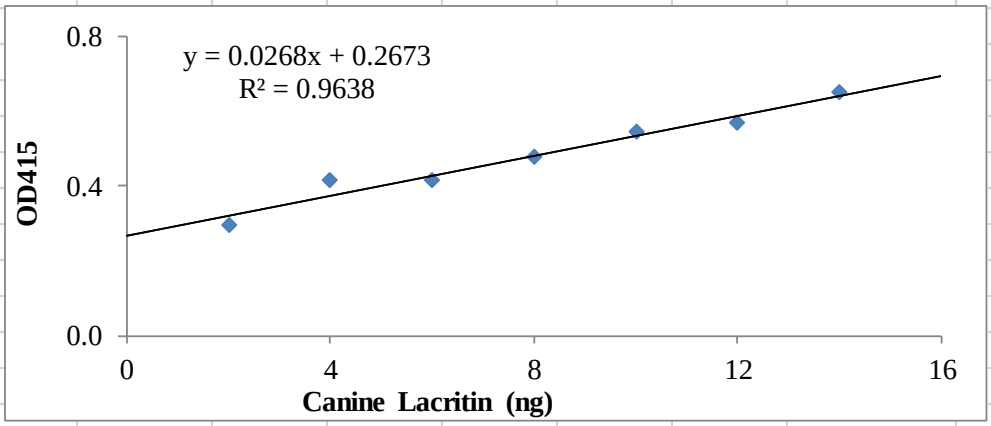
ng cLac	abs 1	abs 2	abs 3	average
16	0.886	0.775	0.867	
14	0.662	0.627	0.662	0.650
12	0.582	0.565	0.560	0.569
10	0.515	0.541	0.576	0.544
8	0.479	0.451	0.506	0.479
6	0.401	0.414	0.438	0.418
4	0.397	0.434	0.416	0.416
2	0.281	0.298	0.306	0.295
0	0.059	0.057	0.056	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 039	0.406	0.412	0.468	0.429	0.034	6.021	DE	Yes
OS 039	0.184	0.189	0.199	0.191	0.008	-2.859	DE	Yes
OD 18	0.061	0.067	0.071	0.066	0.005	-7.499	DE	No
OS 18	0.136	0.129	0.136	0.134	0.004	-4.986	DE	No
OD 100	0.656	0.808	0.763	0.742	0.078	17.725	DE	No
OS 100	0.846	0.647	0.517	0.670	0.166	15.026	N	
OD 30	0.745	0.697	0.740	0.727	0.026	17.165	N	
OS 30	0.778	0.649	0.726	0.718	0.065	16.805	N	

Standard Curve 2-11-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

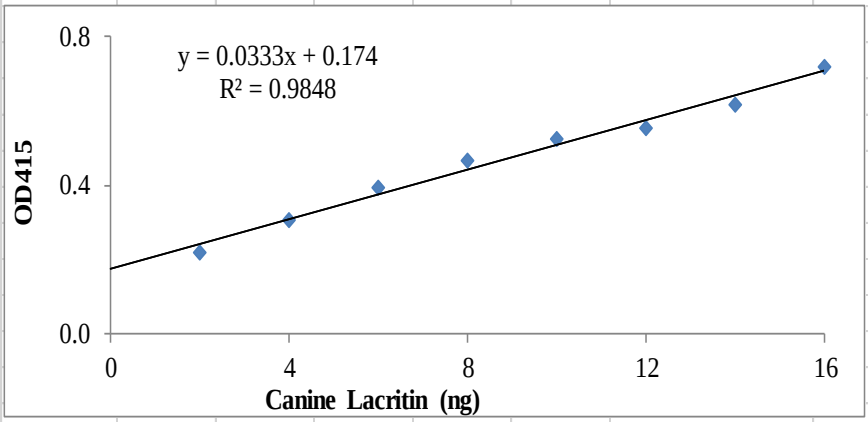
ng cLac	abs 1	abs 2	abs 3	average
16	0.886	0.775	0.867	
14	0.662	0.627	0.662	0.650
12	0.582	0.565	0.560	0.569
10	0.515	0.541	0.576	0.544
8	0.479	0.451	0.506	0.479
6	0.401	0.414	0.438	0.418
4	0.397	0.434	0.416	0.416
2	0.281	0.298	0.306	0.295
0	0.059	0.057	0.056	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 039	0.406	0.412	0.468	0.429	0.034	6.021	DE	Yes
OS 039	0.184	0.189	0.199	0.191	0.008	-2.859	DE	Yes
OD 18	0.061	0.067	0.071	0.066	0.005	-7.499	DE	No
OS 18	0.136	0.129	0.136	0.134	0.004	-4.986	DE	No
OD 100	0.656	0.808	0.763	0.742	0.078	17.725	DE	No
OS 100	0.846	0.647	0.517	0.670	0.166	15.026	N	
OD 30	0.745	0.697	0.740	0.727	0.026	17.165	N	
OS 30	0.778	0.649	0.726	0.718	0.065	16.805	N	

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

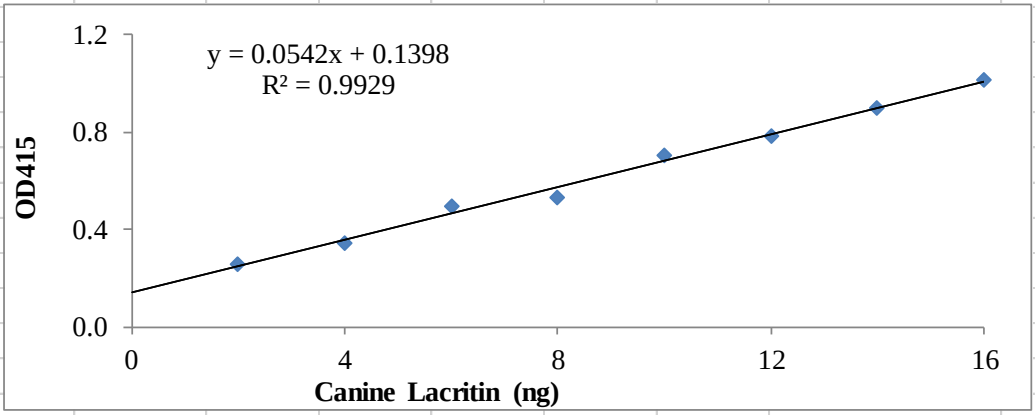
ng cLac	abs 1	abs 2	abs 3	average
16	0.735	0.719	0.700	0.718
14	0.596	0.622	0.637	0.618
12	0.542	0.524	0.588	0.551
10	0.513	0.528	0.536	0.526
8	0.460	0.469	0.462	0.464
6	0.400	0.394	0.384	0.393
4	0.300	0.312	0.307	0.306
2	0.217	0.214	0.221	0.217
0	0.136	0.062	0.092	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?
OS 68	0.199	0.187	0.203	0.203	0.008	0.901	DE
OD 68	0.697	0.728	0.733	0.733	0.020	16.769	DE
OS 43	0.313	0.327	0.333	0.333	0.010	4.793	DE
OD 43	0.361	0.375	0.362	0.362	0.008	5.662	DE
OS 91	0.293	0.307	0.290	0.290	0.009	3.506	N
OD 91	0.774	0.795	0.748	0.748	0.024	17.219	N
OS 03	0.846	0.821	0.825	0.825	0.013	19.524	N
OD 03	0.327	0.329	0.315	0.315	0.008	4.254	N

Standard Curve 2-11-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

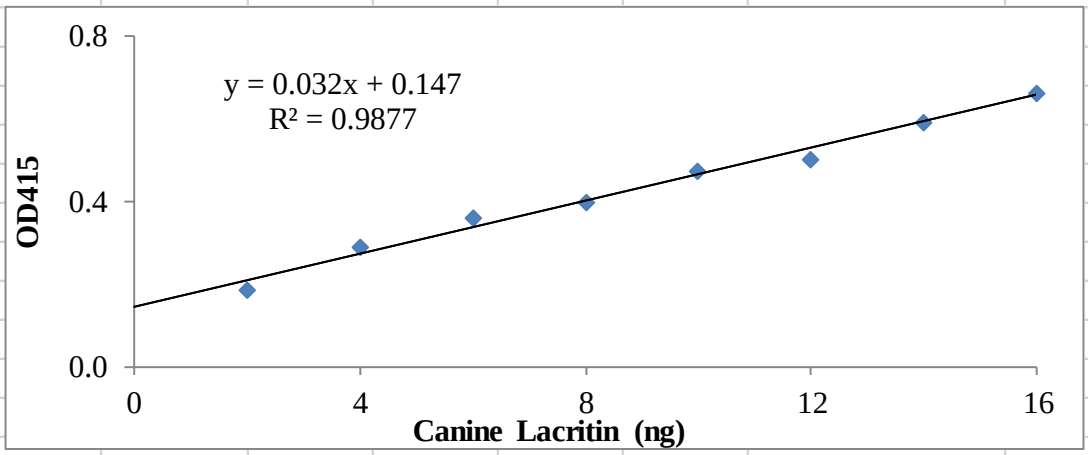
ng cLac	abs 1	abs 2	abs 3	average
16	1.036	1.031	0.968	1.012
14	0.897	0.869	0.929	0.898
12	0.754	0.742	0.846	0.781
10	0.703	0.706	0.702	0.704
8	0.541	0.524	0.534	0.533
6	0.475	0.473	0.542	0.497
4	0.334	0.340	0.347	0.340
2	0.311	0.225	0.231	0.256
0	0.199	0.047	0.057	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?	n treatmen
OD 428	0.478	0.511	0.563	0.517	0.043	6.966	DE	Yes
OS 428	0.185	0.173	0.179	0.179	0.006	0.723	DE	Yes
OD 989	1.003	0.692	1.003	0.899	0.180	14.014	DE	No
OS 989	1.154	1.141	1.103	1.133	0.027	18.319	DE	No
OD 897	1.406	1.326	1.339	1.357	0.043	22.458	N	No
OS 897	1.350	1.311	1.303	1.321	0.025	21.800	N	No
OD 481	1.201	1.201	1.175	1.192	0.015	19.419	N	No
OS 481	1.247	1.201	1.316	1.255	0.058	20.569	N	No

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

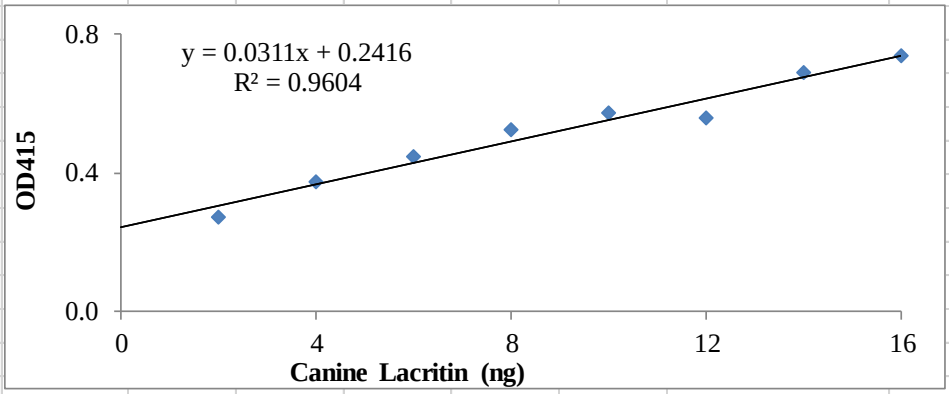
ng cLac	abs 1	abs 2	abs 3	average
16	0.647	0.673	0.677	0.666
14	0.581	0.596	0.600	0.592
12	0.491	0.507	0.517	0.505
10	0.473	0.486	0.462	0.474
8	0.382	0.395	0.427	0.401
6	0.352	0.368	0.369	0.363
4	0.275	0.294	0.298	0.289
2	0.181	0.189	0.190	0.187
0	0.048	0.049	0.050	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?
OS 68	0.167	0.191	0.162	0.173	0.016	0.823	DE
OD 68	0.652	0.585	0.647	0.628	0.037	15.031	DE
OS 43	0.293	0.279	0.296	0.289	0.009	4.448	DE
OD 43	0.334	0.336	0.329	0.333	0.004	5.813	DE
OS 91	0.274	0.262	0.281	0.272	0.010	3.917	N
OD 91	0.706	0.607	0.650	0.654	0.050	15.854	N
OS 03	0.785	0.772	0.782	0.780	0.007	19.771	N
OD 03	0.259	0.284	0.297	0.280	0.019	4.156	N

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

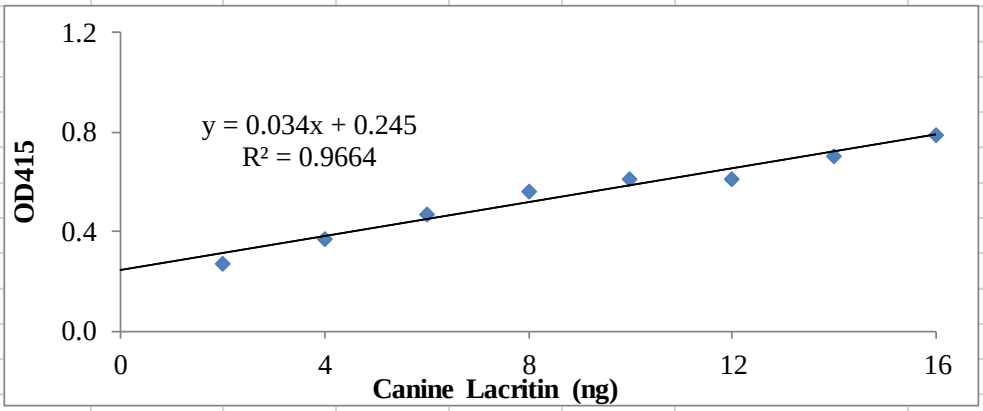
ng cLac	abs 1	abs 2	abs 3	average
16	0.702	0.730	0.783	0.738
14	0.674	0.691	0.695	0.687
12	0.543	0.538	0.588	0.556
10	0.581	0.556	0.576	0.571
8	0.532	0.522	0.522	0.525
6	0.468	0.432	0.443	0.448
4	0.360	0.379	0.382	0.374
2	0.265	0.266	0.278	0.270
0	0.058	0.062	0.059	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?	On treatment?
OD 839	0.489	0.415	0.512	0.472	0.051	7.408	DE	Yes
OS 839	0.633	0.657	0.659	0.650	0.014	13.121	DE	Yes
OS 581	0.364	0.372	0.392	0.376	0.014	4.322	DE	No
OS 570	0.731	0.747	0.751	0.743	0.011	16.122	N	No
OD 478	0.749	0.761	0.756	0.755	0.006	16.519	N	No
OS 478	0.638	0.669	0.662	0.656	0.016	13.335	N	No
OD 415	0.975	0.991	1.029	0.998	0.028	24.332	N	No
OS 415	0.959	0.946	0.996	0.967	0.026	23.325	N	No

Standard Curve 2-18-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

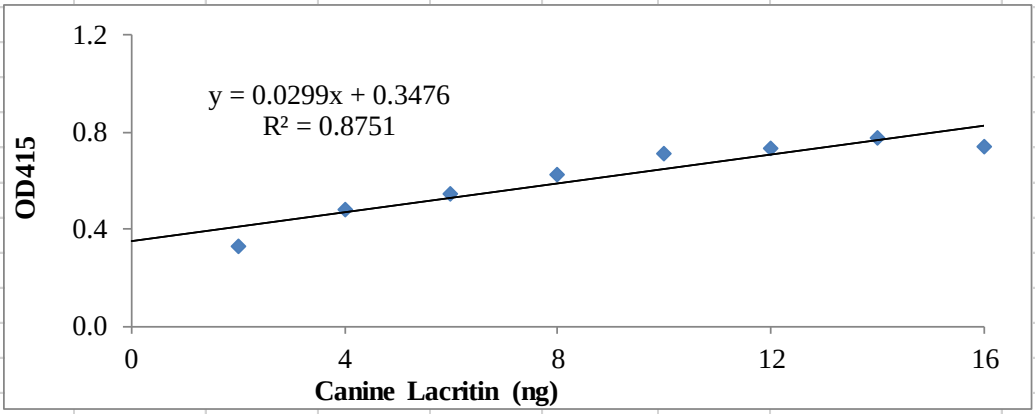
ng cLac	abs 1	abs 2	abs 3	average
16	0.772	0.779	0.812	0.788
14	0.684	0.714	0.730	0.709
12	0.577	0.638	0.623	0.613
10	0.577	0.603	0.654	0.611
8	0.548	0.540	0.612	0.567
6	0.477	0.467	0.470	0.471
4	0.373	0.367	0.383	0.374
2	0.262	0.288	0.281	0.277
0	0.052	0.051	0.051	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	n treatmen
OD 839	0.521	0.498	0.560	0.526	0.031	8.275	DE	Yes
OS 839	0.691	0.725	0.727	0.714	0.020	13.804	DE	Yes
OS 581	0.360	0.401	0.377	0.379	0.021	3.951	DE	No
OS 570	0.750	0.737	0.735	0.741	0.008	14.578	N	No
OD 478	0.806	0.786	0.780	0.791	0.014	16.049	N	No
OS 478	0.703	0.630	0.665	0.666	0.037	12.382	N	No
OD 415	1.065	1.091	1.077	1.078	0.013	24.490	N	No
OS 415	1.012	0.993	0.985	0.997	0.014	22.108	N	No

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

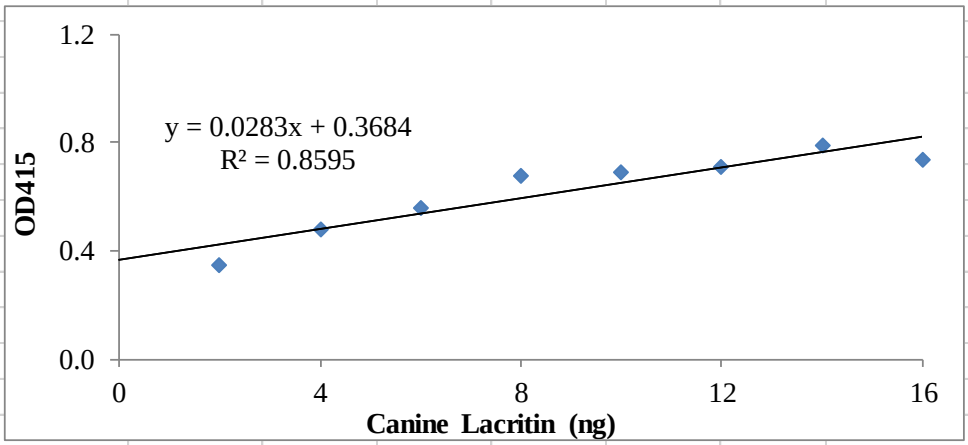
ng cLac	abs 1	abs 2	abs 3	average
16	0.705	0.747	0.758	0.737
14	0.760	0.773	0.802	0.778
12	0.684	0.746	0.766	0.732
10	0.696	0.714	0.721	0.710
8	0.613	0.632	0.628	0.624
6	0.565	0.536	0.542	0.548
4	0.457	0.490	0.484	0.477
2	0.308	0.333	0.337	0.326
0	0.051	0.058	0.057	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?	n treatmen
OD 564	0.420	0.438	0.468	0.442	0.013	3.157	DE	Y
OS 183	0.378	0.365	0.390	0.378	0.013	1.006	DE	N
OD 803	0.681	0.699	0.756	0.712	0.074	12.187	N	
OS 803	0.741	0.829	0.769	0.780	0.049	14.450	N	
OD 0039	0.718	0.674	0.651	0.681	0.062	11.151	N	
OS 0039	0.848	0.774	0.813	0.812	0.070	15.521	N	
OD 477	0.952	0.948	0.923	0.941	0.052	19.846	N	
OS 477	0.865	0.851	0.814	0.843	0.036	16.580	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

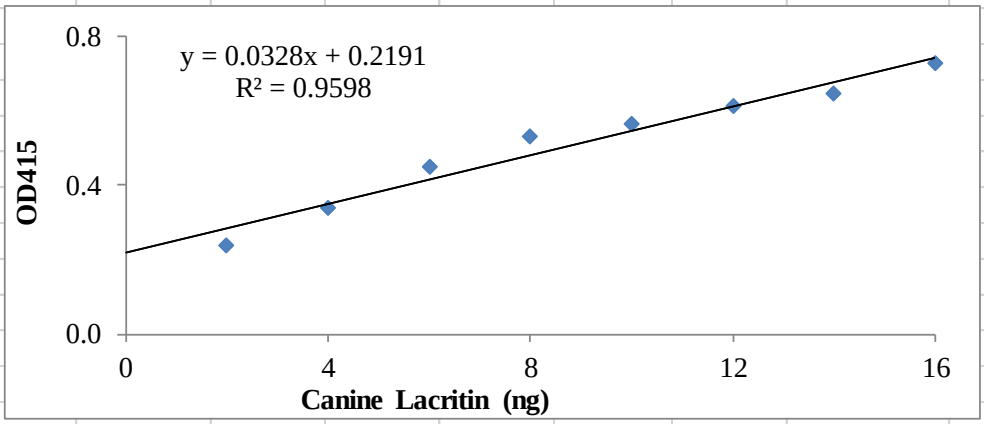
ng cLac	abs 1	abs 2	abs 3	average
16	0.695	0.754	0.766	0.738
14	0.757	0.768	0.840	0.788
12	0.687	0.676	0.759	0.707
10	0.654	0.697	0.724	0.692
8	0.638	0.625	0.765	0.676
6	0.569	0.528	0.569	0.555
4	0.461	0.486	0.492	0.480
2	0.336	0.351	0.355	0.347
0	0.050	0.06	0.058	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 564	0.476	0.433	0.498	0.469	0.033	3.555	DE	Y
OS 183	0.404	0.392	0.410	0.402	0.009	1.187	DE	N
OD 803	0.808	0.769	0.741	0.773	0.034	14.285	N	
OS 803	0.806	0.803	0.848	0.819	0.025	15.922	N	
OD 0039	0.771	0.786	0.777	0.778	0.008	14.473	N	
OS 0039	0.848	0.843	0.793	0.828	0.030	16.240	N	
OD 477	0.968	1.007	0.889	0.955	0.060	20.716	N	
OS 477	0.969	0.862	0.835	0.889	0.071	18.384	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

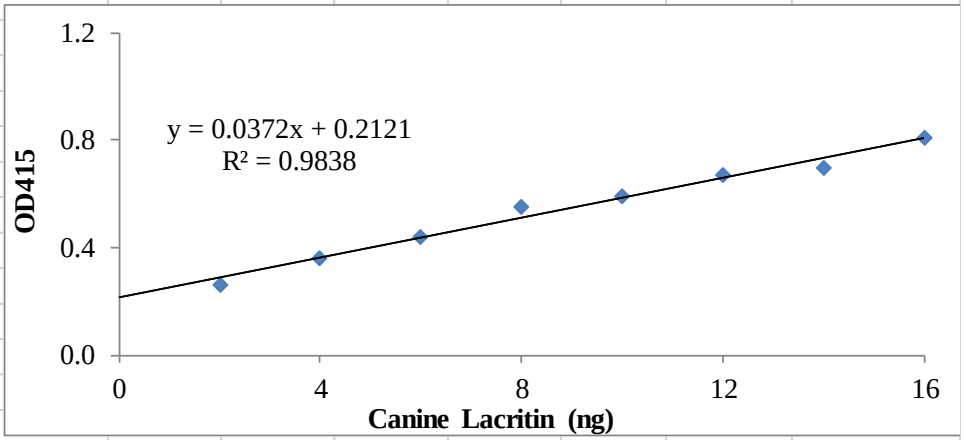
ng cLac	abs 1	abs 2	abs 3	average
16	0.707	0.736	0.748	0.730
14	0.637	0.671	0.630	0.646
12	0.528	0.593	0.716	0.612
10	0.537	0.573	0.583	0.564
8	0.535	0.526	0.532	0.531
6	0.445	0.447	0.459	0.450
4	0.342	0.336	0.342	0.340
2	0.229	0.238	0.244	0.237
0	0.058	0.054	0.054	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 35	0.146	0.111	0.109	0.122	0.025	-2.960	DE	Y
OS 35	0.112	0.099	0.088	0.100	0.012	-3.641	DE	Y
OD 26	0.477	0.456	0.460	0.464	0.021	7.477	DE	Y
OS 26	0.456	0.502	0.465	0.474	0.272	7.782	DE	Y
OD 47	0.925	0.931	0.867	0.908	0.325	20.993	N	
OS 47	0.341	0.336	0.308	0.328	0.066	3.330	N	
OD 139	0.219	0.213	0.177	0.203	0.176	-0.491	N	
OS 139	0.597	0.500	0.573	0.557	0.017	10.292	DE	Y
OD 747	0.903	0.89	0.897	0.897	0.004	20.658	N	
OS 747	0.695	0.704	0.661	0.687	0.024	14.255	N	
OD 415	0.857	0.81	0.763	0.810	0.066	18.015	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

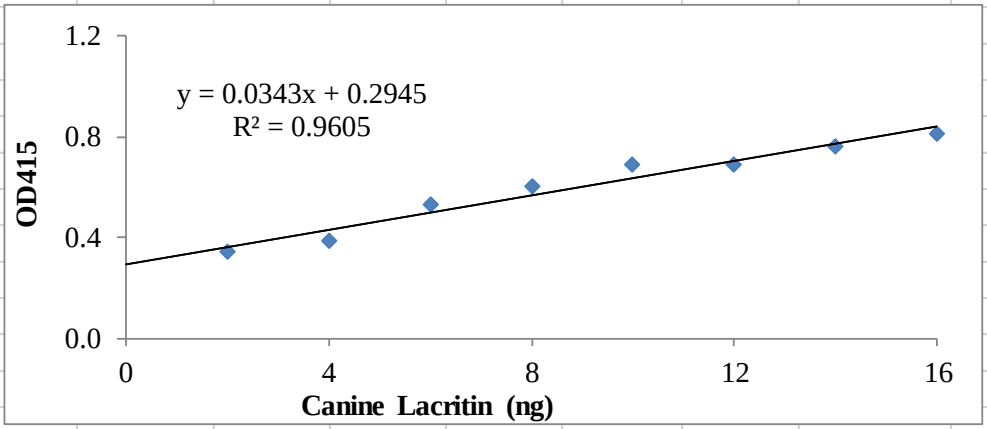
ng cLac	abs 1	abs 2	abs 3	average
16	0.804	0.786	0.829	0.806
14	0.667	0.732	0.698	0.699
12	0.621	0.674	0.709	0.668
10	0.584	0.574	0.607	0.588
8	0.528	0.534	0.597	0.553
6	0.459	0.415	0.440	0.438
4	0.349	0.359	0.370	0.359
2	0.245	0.27	0.267	0.261
0	0.050	0.052	0.051	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 35	0.127	0.108	0.115	0.117	0.010	-2.565	DE	Y
OS 35	0.100	0.083	0.093	0.092	0.009	-3.228	DE	Y
OD 26	0.385	0.423	0.484	0.431	0.050	5.875	DE	Y
OS 26	0.447	0.459	0.466	0.457	0.010	6.592	DE	Y
OD 47	1.048	0.975	0.943	0.989	0.054	20.875	N	
OS 47	0.348	0.337	0.321	0.335	0.014	3.313	N	
OD 139	0.208	0.195	0.215	0.206	0.010	-0.164	N	
OS 139	0.579	0.530	0.533	0.547	0.027	9.012	DE	Y
OD 747	0.963	0.907	0.921	0.930	0.029	19.307	N	
OS 747	0.736	0.675	0.721	0.711	0.032	13.402	N	
OD 415	0.896	0.8	0.794	0.830	0.057	16.610	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

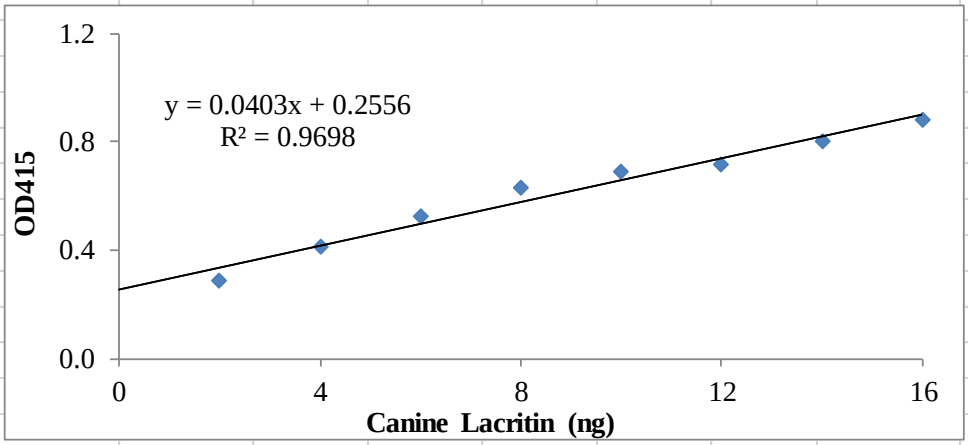
ng cLac	abs 1	abs 2	abs 3	average
16	0.779	0.836	0.830	0.815
14	0.733	0.750	0.808	0.764
12	0.646	0.648	0.781	0.692
10	0.636	0.692	0.746	0.691
8	0.601	0.606	0.599	0.602
6	0.522	0.509	0.553	0.528
4	0.379	0.391	0.398	0.389
2	0.362	0.379	0.287	0.343
0	0.157	0.050	0.054	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	ng per 50 ng	Dry eye?	On treatment?
OD 564	0.351	0.378	0.336	0.355	0.019	1.764	DE	Y
OS 185	0.170	0.161	0.172	0.168	0.006	-3.698	DE	N
OD 803	0.660	0.671	0.674	0.668	0.066	10.899	N	
OS 803	0.748	0.780	0.724	0.751	0.049	13.299	N	
OD 0039	0.702	0.654	0.634	0.663	0.060	10.753	N	
OS 0039	0.788	0.753	0.751	0.764	0.083	13.688	N	
OD 477	1.010	0.910	0.918	0.946	0.123	18.994	N	
OS 477	0.838	0.767	0.787	0.797	0.036	14.660	N	
OD 100	0.43	0.416	0.403	0.416	0.019	3.552	DE	N
OS 303	0.253	0.255	0.258	0.255	0.004	-1.142	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

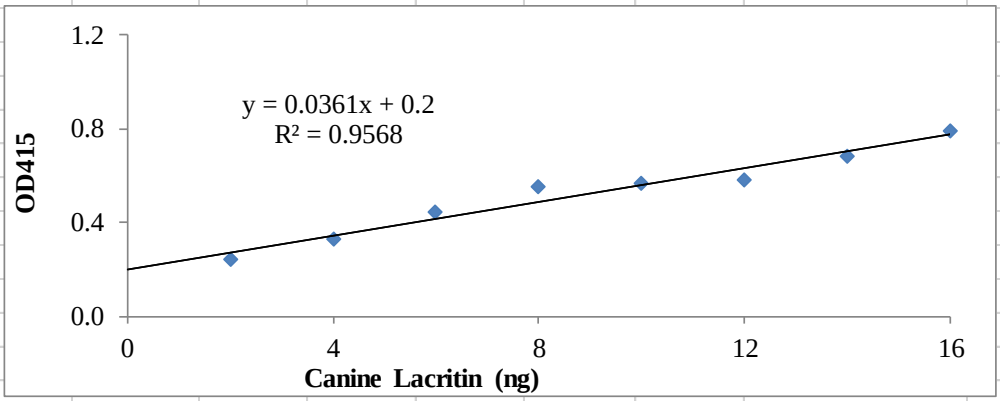
ng cLac	abs 1	abs 2	abs 3	average
16	0.863	0.866	0.918	0.882
14	0.775	0.811	0.825	0.804
12	0.689	0.735	0.727	0.717
10	0.692	0.691	0.695	0.693
8	0.627	0.646	0.627	0.633
6	0.515	0.522	0.529	0.522
4	0.395	0.419	0.425	0.413
2	0.279	0.281	0.296	0.285
0	0.049	0.052	0.051	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OD 564	0.344	0.304	0.314	0.321	0.021	1.615	DE	Y
OS 185	0.177	0.167	0.179	0.174	0.006	-2.017	DE	N
OD 803	0.678	0.639	0.714	0.677	0.038	10.457	N	
OS 803	0.744	0.781	0.736	0.754	0.024	12.359	N	
OD 0039	0.675	0.666	0.636	0.659	0.020	10.010	N	
OS 0039	0.733	0.641	0.707	0.694	0.047	10.870	N	
OD 477	0.922	0.883	0.889	0.898	0.021	15.940	N	
OS 477	0.777	0.801	0.779	0.786	0.013	13.153	N	
OD 100	0.386	0.413	0.377	0.392	0.019	3.385	DE	N
OS 303	0.243	0.238	0.222	0.234	0.011	-0.528	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

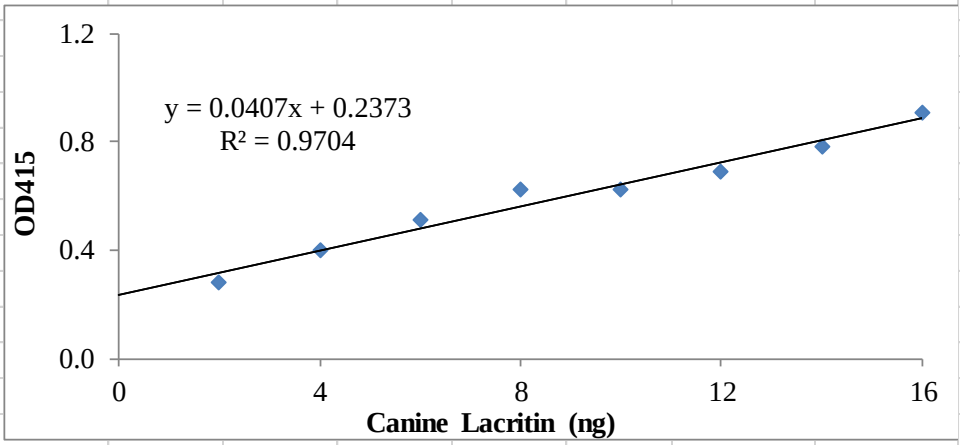
ng cLac	abs 1	abs 2	abs 3	average
16	0.793	0.781	0.808	0.794
14	0.670	0.688	0.688	0.682
12	0.557	0.587	0.607	0.584
10	0.556	0.551	0.600	0.569
8	0.530	0.542	0.593	0.555
6	0.434	0.439	0.466	0.446
4	0.309	0.334	0.350	0.331
2	0.229	0.243	0.245	0.239
0	0.052	0.051	0.054	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OS 35	0.265	0.250	0.271	0.262	0.011	1.717	DE	Y
OD 139	0.294	0.262	0.291	0.282	0.018	2.281	N	
OS 139	0.671	0.679	0.755	0.702	0.110	13.897	DE	Y
OD 826	0.542	0.537	0.519	0.533	0.090	9.215	DE	Y
OS 826	0.412	0.376	0.383	0.390	0.084	5.272	DE	Y
OD 100	0.577	0.540	0.542	0.553	0.048	9.778	DE	N
OS 303	0.668	0.637	0.625	0.643	0.122	12.281	N	
OD 415	0.860	0.855	0.800	0.838	0.042	17.682	N	
OD 747	0.74	0.71	0.654	0.701	0.061	13.887	N	
OS 747	0.535	0.51	0.531	0.525	0.003	9.012	N	

Standard Curve 2-29-16: cLAC 1/22/16, 6924 FB PANT (1:6400), Secondary (1:1200)

ng cLac	abs 1	abs 2	abs 3	average
16	0.882	0.941	0.907	0.910
14	0.785	0.765	0.801	0.784
12	0.698	0.681	0.690	0.690
10	0.614	0.609	0.652	0.625
8	0.629	0.608	0.630	0.622
6	0.514	0.517	0.512	0.514
4	0.392	0.399	0.405	0.399
2	0.287	0.283	0.282	0.284
0	0.054	0.053	0.052	



Sample	ABS 1	ABS 2	ABS 3	AVG	ST DEV	g per 50 n	Dry eye?	On treatment?
OS 35	0.295	0.284	0.287	0.289	0.006	1.262	DE	Y
OD 139	0.326	0.336	0.339	0.334	0.007	2.368	N	
OS 139	0.789	0.831	0.800	0.807	0.022	13.989	DE	Y
OD 826	0.598	0.604	0.573	0.592	0.016	8.707	DE	Y
OS 826	0.444	0.474	0.437	0.452	0.020	5.267	DE	Y
OD 100	0.649	0.639	0.608	0.632	0.021	9.698	DE	N
OS 303	0.727	0.708	0.663	0.699	0.033	11.352	N	
OD 415	0.920	0.930	0.876	0.909	0.029	16.495	N	
OD 747	0.794	0.79	0.734	0.773	0.034	13.154	N	
OS 747	0.601	0.602	0.603	0.602	0.001	8.961	N	

APPENDIX C

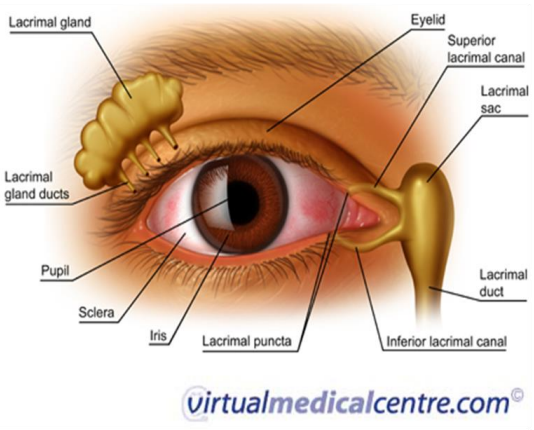
Below is the presentation that will be given at the ISAT Senior Symposium on April 15th, 2016.

Clinical Study of Canine Tear Lacritin as a Treatment for Dry Eye

KATIE KELLY

ISAT SENIOR CAPSTONE, HONORS THESIS
ISAT SENIOR CAPSTONE SYMPOSIUM, APRIL 15TH, 2016
DEPARTMENT OF INTEGRATED SCIENCE AND ENGINEERING

Lacritin

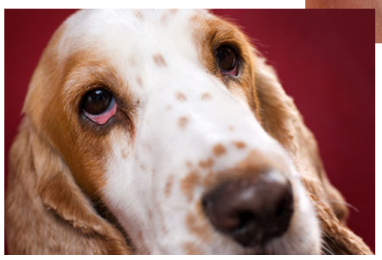


Gordon Laurie, University of Virginia

- What is lacritin?
- Why are we interested in it?
- Formation of a Lacritin Consortium

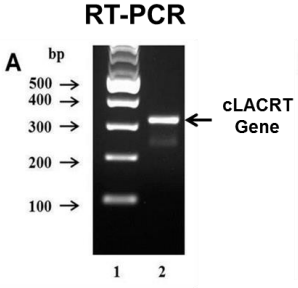
Dry Eye

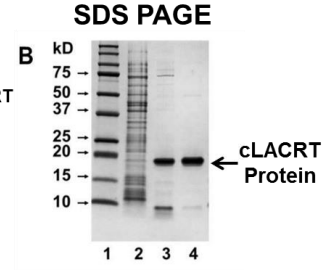
- Affects both humans and canines
- Underdiagnosed and poorly understood
- Life long condition
- Affects 25 million Americans and 4% of the canine population

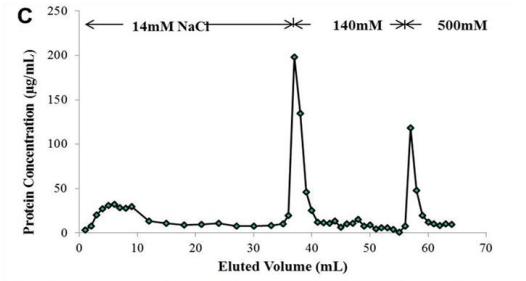


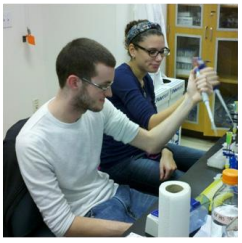
Canine Project Previous Work

- Cloning
- Expression and Purification
- Antibody Production







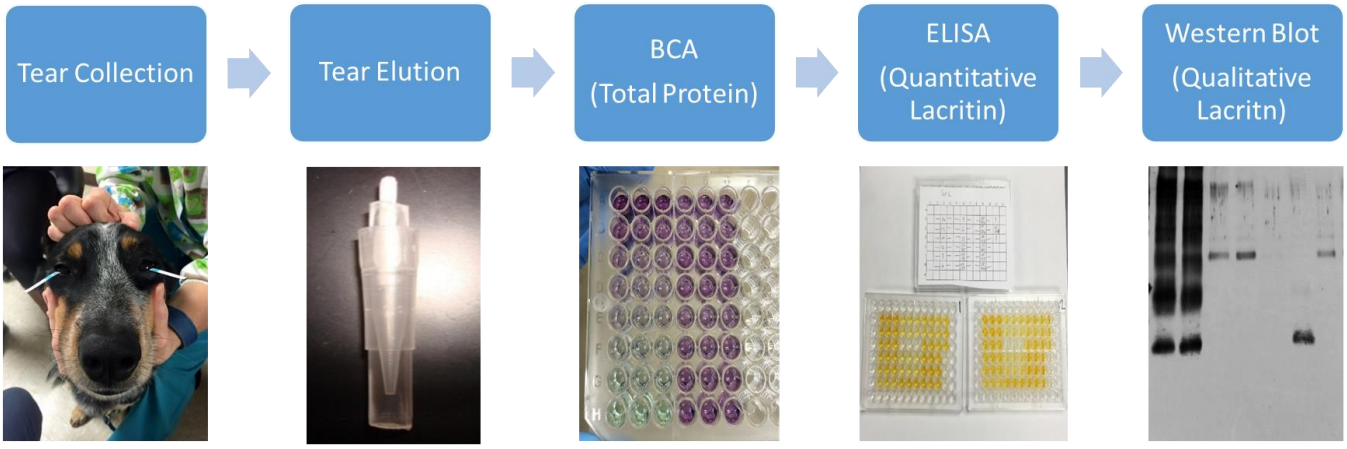


My Project

Serves Two Purposes:

- Animal Model System for a Potential Human Therapeutic
- Potential Canine Therapeutic

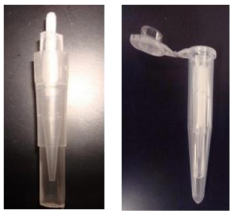
Process Flow

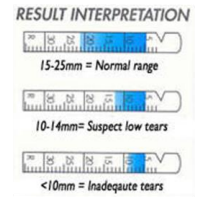


Tear Collection and Processing

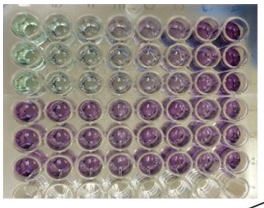
Schirmer Tear Test (STT) and Tear Collection

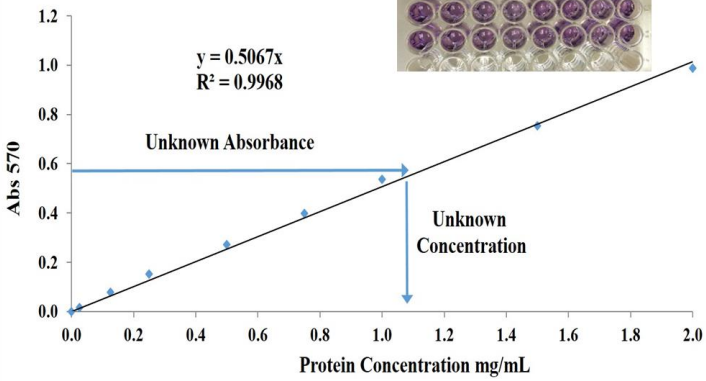
Tear Elution



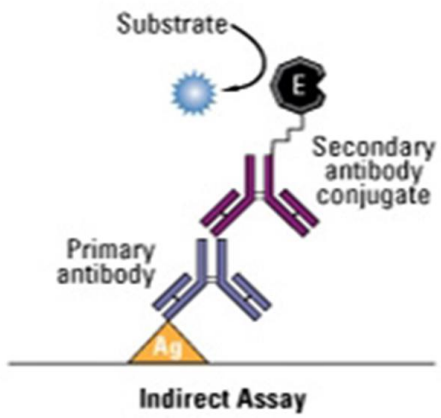


BCA Assay Total Protein





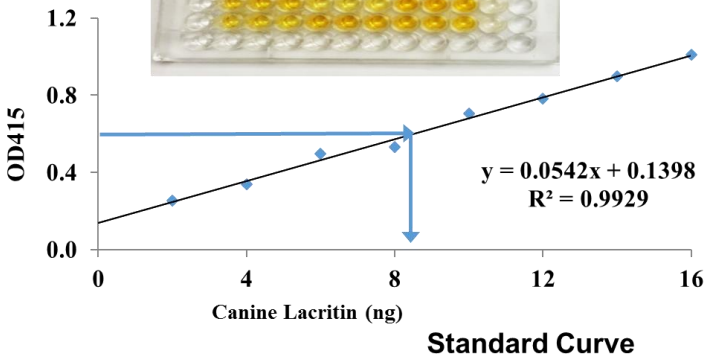
Indirect ELISA Development



Indirect Assay

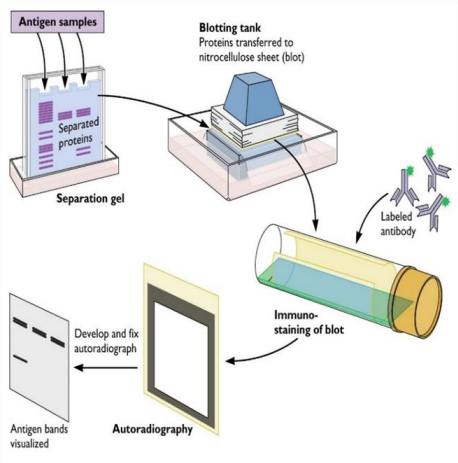
Antibody Configuration for Indirect ELISA

Developed Plate



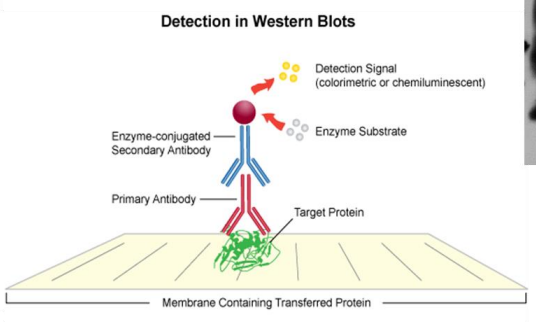
Standard Curve

Western Blots

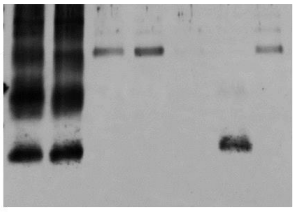


Process for Western Blots

Antibody configuration similar to ELISA

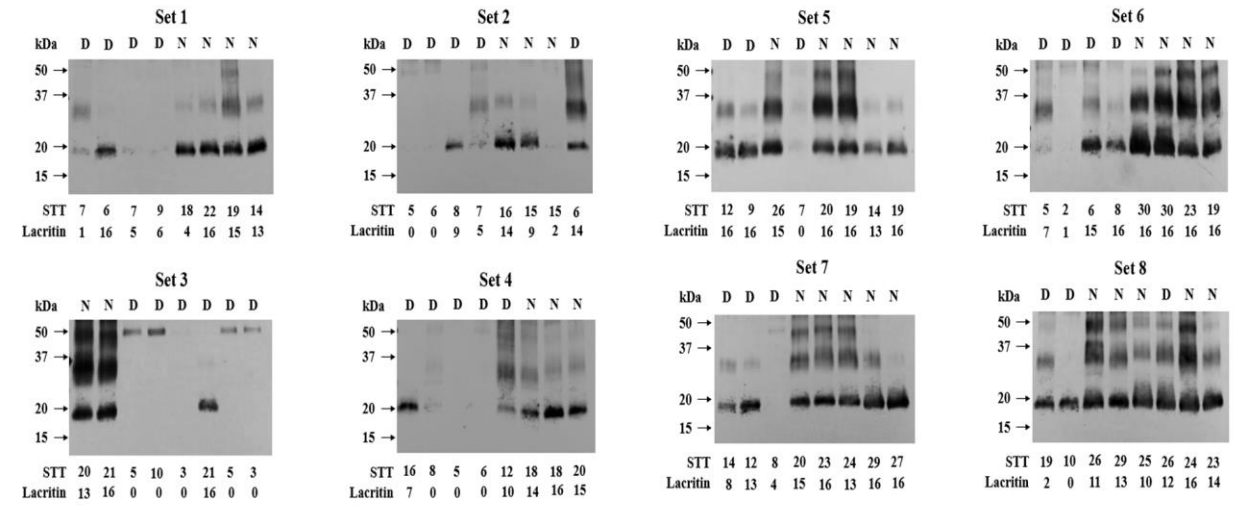


Developed Western Blot



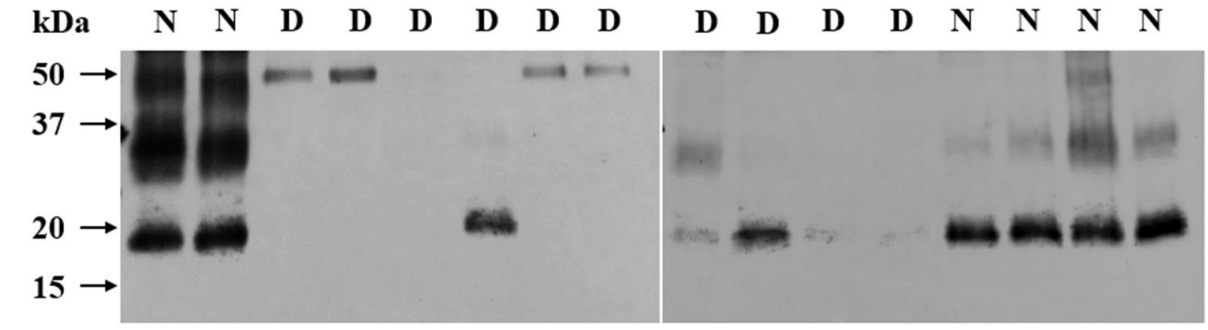
Detects only Lacritin proteins

Western Blot Analysis



32 Normal Tear Samples (N) and 32 Dry Eye Tear Samples (D)

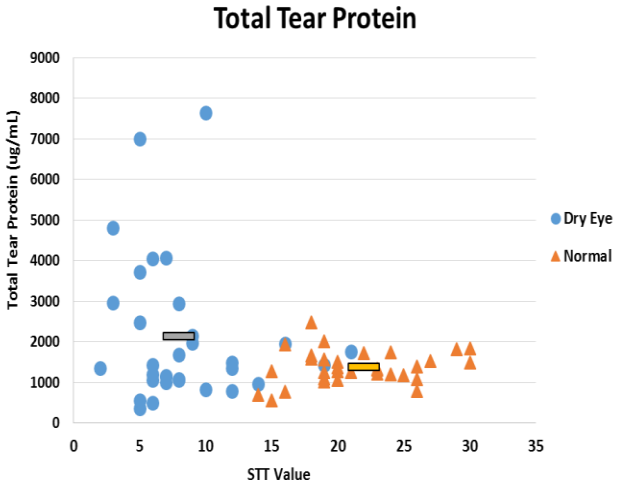
Western Blot and ELISA Analysis

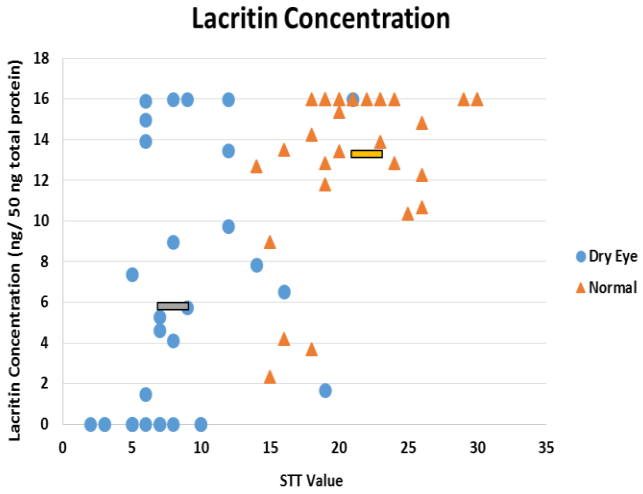


STT	20	21	5	10	3	21	5	3	7	6	7	9	18	22	19	14
Lacritin	13	16	0	0	0	16	0	0	1	16	5	6	4	16	15	13

N = Normal sample, D = Dry Eye sample STT = Schirmer Tear Test
Lacritin = ng/50 ng total protein by ELISA

What does it all mean?





Conclusions

- Developed a working diagnostic assay for lacritin in canine tears
- Used the process to analyze 64 clinical tear samples
- Found that lacritin was down regulated in dry eye tear samples

Summary of Lacritin Concentrations in 64 Samples

Sample	Range	Average
Dry Eye	0-16 ng	5.8 ng
Normal	2.32- 16 ng	13.3 ng

Next Steps

- Topical application in canines
- Human clinical trials
- Ultimate goal: bring drug to market

FDA APPROVED

Thank You!!

- Dr. McKown
- Dr. Raab and Dr. Stockwell
- Dr. Julie Disney
- Eliza Gaylord and lab mates
- Family and Friends

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Questions?

Thanks for listening!