

Spring 2014

# Canine clinical study for tear lacritin as a treatment for dry eye

Alan Chancellor Tate  
*James Madison University*

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

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A Project Presented to

the Faculty of the Undergraduate

Colleges of Integrated Science and Engineering & Science and Mathematics

James Madison University

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in Partial Fulfillment of the Requirements

for the Degree of Bachelor of Science

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by Alan Chancellor Tate

May 2014

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Accepted by the faculty of the Departments of Integrated Science and Technology & Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

FACULTY COMMITTEE:

HONORS PROGRAM APPROVAL:

---

Project Advisor: Robert L. McKown, Ph.D.,  
Professor, ISAT

---

Barry Falk, Ph.D.,  
Director, Honors Program

---

Reader: Ronald Raab, Ph.D.,  
Professor, ISAT

---

Reader: Kyle Seifert, Ph.D.,  
Associate Professor, Biology

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## **Preface**

All research presented in this thesis was performed by the primary author over the course of one year and is a continuing body of work. When other work is presented, the researcher is noted.

## **Acknowledgements**

I would like to thank my advisor, Dr. Robert McKown, for providing me with the opportunity to perform research under his guidance at James Madison University. Dr. McKown has shown me kindness in every way and has been an irreplaceable mentor during my time here at this institution. I would also like to thank the fellow students, faculty, and staff members that have assisted me in completing my honors capstone project. I am extremely grateful for the contributions made by Cara Soyars and Allie Enghauser. I would also like to give additional thanks to my readers, Dr. Ronald Raab and Dr. Kyle Seifert for reviewing my thesis and offering valuable guidance. I would also like to recognize this work was supported by the 4-VA Collaborative at James Madison University. I would like to give special thanks and recognition to my family and friends for their support, guidance, and love throughout my educational career.

## Abstract

**Purpose:** Lacritin is a human tear protein first characterized as a novel secretion enhancing factor from the human lacrimal gland (Sanghi, *et. al.*, 2001). Preclinical animal studies have shown that recombinant human lacritin promotes basal tearing in rabbit eyes upon topical application (Samudre *et. al.*, 2011). Antibodies were produced specific for human lacritin, and a clinical assay was developed to detect and quantify lacritin in human tear samples (Seifert K, *et. al.*, 2012). Lacritin is currently being developed as a new topical therapeutic for the treatment of dry eye; however, a dry eye animal model system is needed to test lacritin prior to human clinical trials. The canine dry eye model is promising in that it is a common naturally occurring disorder with clear clinical parallels to dry eye disease in humans (Kymionis, *et. al.*, 2008). The first objective of the study was to determine if the assay to detect lacritin in human tears could detect a lacritin like protein in canine tears. Then an effort was made to clone, sequence, and purify the canine lacritin protein. The purified canine lacritin will be used for a preclinical study in a healthy canine population to test for stimulation of tear production upon topical application.

**Methods:** Fresh canine lacrimal glands were collected at VA-MD Regional College of Veterinary Medicine. RT-PCR was used to amplify the canine lacritin gene from lacrimal tissue. The gene was cloned into an intein expression vector and sequenced. Canine lacritin was expressed in *E. coli*, purified by chitin and DEAE chromatography, and analyzed by SDS PAGE.

**Results:** A canine lacritin gene homolog was amplified from lacrimal tissue and sequenced. The canine lacritin gene was expressed in *E. coli* and purified to a single protein band on SDS PAGE.

**Conclusions:** The gene for canine lacritin has been successfully cloned and sequenced. Protein sequence analysis reveals the canine lacritin is 29% identical to human lacritin. The canine lacritin DNA has been expressed in *E. coli*, and purified to greater than 95% purity.

## Introduction

### Discovery and Function of Lacritin

Discovered in 2001 by Dr. Gordon Laurie of the University of Virginia, lacritin is a human tear glycoprotein (Sanghi, *et. al.*, 2001) that is mitogenic, prosecretory (Wang, *et. al.*, 2006), and has recently been shown to possess bactericidal activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermis* (McKown RL, *et al.* IOVS 2010;51:ARVO E-Abstract 4181). This 12.3 kDa protein is comprised of 119 amino acids after cleavage of the 19 amino acid N-terminal signal sequence during secretion from the lacrimal gland (Ma, *et. al.*, 2008).

The discovery of this novel protein resulted in the organization of a lacritin research consortium. Researchers from University of Virginia, James Madison University, Eastern Virginia Medical School, Fort Belvoir Community Hospital, and most recently Virginia Polytechnic Institute form this consortium. The purpose of this consortium has since been to thoroughly investigate and characterize lacritin in hopes to develop a new human biological therapeutic.

Lacritin is the only prosecretory protein that has been shown to be down regulated in patients with dry eye (Srinivasan, *et. al.*, 2012). It is produced by human acinar cells within the secretory granules of the lacrimal gland, and is released to the surface of the eye within tears through ducts (Ma, *et. al.*, 2008). Findings have revealed that lacritin can induce production and stimulate secretion after binding to corneal epithelial cells (Sanghi, *et. al.*, 2001). Furthermore, it has been shown that upon topical application of lacritin to the eyes of rabbits, basal tearing is promoted (Samudre *et. al.*, 2011). The expression of lacritin, or a similar ortholog, has currently



been documented in humans (Sanghi, *et. al.*, 2001), non-human primates (Nakajima, *et. al.*, 2007), and also horses (Laurie, *et. al.*, 2012).

### **The Purpose of a Canine Lacritin Study**

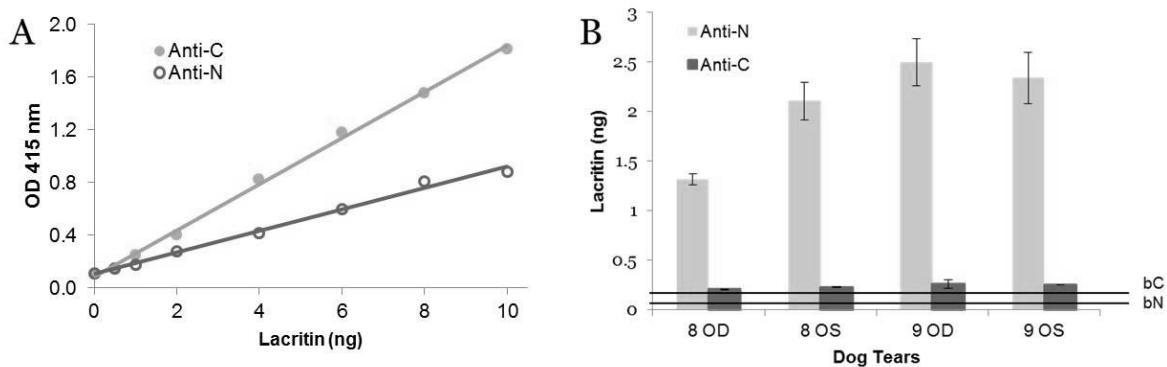
The health and function of the cornea is maintained through the presence of tears (Ofri, *et. al.*, 2009). Keratoconjunctivitis sicca (KCS), the deficiency of tears commonly referred to as dry eye syndrome, has been observed throughout the human species (Moss, *et. al.*, 2000). Almost 25% of patients visiting ophthalmic clinics (Ofri, *et. al.*, 2009) and more than 10% of adults 65 and older (Schein, *et. al.*, 1997) were found to experience the symptoms of dry eye syndrome. KCS has also been observed in 1.5% of canines visiting veterinary hospitals (Ofri, *et. al.*, 2009). Canine KCS may be acute or chronic, and clinical drugs have been tested and developed to treat this syndrome (Reddan, 2011). RESTASIS<sup>®</sup> (cyclosporine ophthalmic emulsion), a therapeutic approved by the FDA for human use (Kymionis, *et. al.*, 2008), has been used in cases of canine KCS in comparison studies with new dry eye therapeutic drugs (Ofri, *et. al.*, 2009). Therefore, the successful treatment of canine KCS with a new therapeutic may provide an acceptable model system for pre-clinical animal trials, leading to human clinical trials.

### **The Exploration of Canine Lacritin**

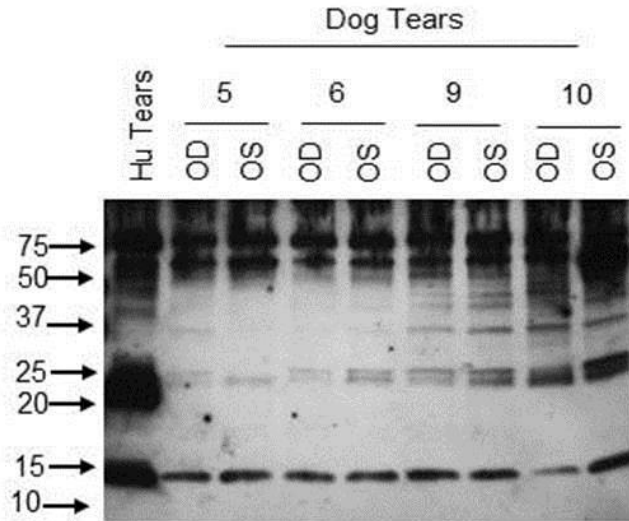
The human *LACRT* gene has been aligned with canine chromosome 27 using the genomic search tool Ensembl ([http://useast.ensembl.org/Homo\\_sapiens/Location/Compara\\_Alignments?align=602&db=core&g=ENSG00000135413&r=12%3A55024595-55028679](http://useast.ensembl.org/Homo_sapiens/Location/Compara_Alignments?align=602&db=core&g=ENSG00000135413&r=12%3A55024595-55028679)). Clustal W2 analysis has revealed that the proposed gene encoding a canine lacritin ortholog is 368



Polyclonal rabbit antibodies have been developed against the 19 amino acid human lacritin N-terminal peptide EDASSDSTGADPAQEAGTS and the recombinant human lacritin N-65 truncation mutant (Seifert, *et. al.*, 2012). These antibodies have successfully been used as primary antibodies to detect lacritin-like proteins from both horse (Laurie, *et. al.*, 2012) and human (Seifert, *et. al.*, 2012) tear samples using Western blot analysis and an indirect Enzyme Linked Immunosorbant Assay (ELISA). As a first attempt to detect a canine lacritin ortholog, tear samples were collected by Dr. Ian Herring at the Veterinary Teaching Hospital at Virginia Polytechnic Institute and the available polyclonal antibodies developed against the human tear lacritin peptide were used for detection. Detection of protein was achieved at James Madison University with both an indirect ELISA (Figure 2) and Western blot analysis (Figure 3) using the polyclonal antibodies developed against the 19 amino acid human lacritin N-terminal peptide, but not the polyclonal antibodies developed against the human lacritin N-65 truncation mutant. The cloning, expression, and purification of the canine lacritin ortholog along with development of specific polyclonal antibodies will benefit the potential for future canine clinical studies.



**Figure 2. ELISA standard curve and canine tear sample results.** (A) Example of the recombinant human lacritin standard curve with the polyclonal antibodies developed against the 19 amino acid human lacritin N-terminal peptide and against the human lacritin N-65 truncation mutant. (B) The amount of lacritin in nanograms for canine tear samples determined using the lacritin standard curves.



**Figure 3. Western blot analysis of multiple canine tear samples and a human tear sample.** The blot was incubated with the polyclonal antibodies created against the 19 amino acid human lacritin N-terminal peptide and developed by chemiluminescence.

The 35% amino acid homology between the human and canine lacritin proteins resulted in detection of protein with only the polyclonal antibody developed against the 19 amino acid human lacritin N-terminal peptide (Figure 2). Therefore, the first objective of this proposed research was to clone and sequence the canine lacritin gene from canine lacrimal gland tissue. The second objective was to clone the canine lacritin gene into an *E. coli* expression vector for production of a recombinant canine lacritin protein and purification using an intein-mediated purification system. Polyclonal antibodies specific to the canine lacritin would then be created to develop immunodiagnostic assays for the detection and quantification of canine lacritin in various canine tear samples. Development of recombinant canine lacritin and a diagnostic assay for canine tear lacritin will provide the tools for development of canine lacritin as a new therapeutic for canine KCS and a potential dry eye animal model system as a pathway to human clinical trials.

## **Materials and Methods**

### **Isolation of total RNA from Canine Lacrimal Tissue**

The Qiagen miRNeasy Micro Kit and Protocol (Qiagen Inc.; Valencia, CA) was used for total RNA extraction from canine lacrimal tissue using spin column technology (Appendix A). Canine lacrimal tissue was isolated by Dr. Ian Herring at VA-MD Regional College of Veterinary Medicine from a thirteen year old female spayed Jack Russell Terrier and stored at 4°C in Qiagen RNAlater RNA Stabilization Reagent. The lacrimal tissue was removed and sliced into a 3 mm cube. Seven hundred microliters of QIAzol Lysis Reagent was added to the sample within a 1.5 ml microcentrifuge tube and the sample was disrupted using a plastic homogenizing tool. The homogenate was incubated at room temperature for 5 minutes. One hundred and forty microliters of chloroform was added to the homogenate and this solution was shaken vigorously for 15 seconds then let sit for three minutes. This solution was centrifuged for 15 minutes at 12,000 x g at 4°C. The aqueous phase was transferred to a new microcentrifuge tube and 525 µl of 100% ethanol was added. Seven hundred microliters of this mixture was transferred into an RNeasy MinElute spin column placed into a 2 ml collection tube and centrifuged for 15 seconds at 8,000 x g at 25°C. The flow-through was discarded and 700 µl of Buffer RWT was added to the spin column and centrifuged for 15 seconds at 8,000 x g at 25°C. The flow-through was discarded and 500 µl of Buffer RPE was added to the spin column and centrifuged for 15 seconds at 8,000 x g at 25°C. Again, the flow-through was discarded and 500 µl of 80% ethanol was added to the spin column and centrifuged for 2 minute at 8000 x g at 25°C. The RNeasy MinElute spin column was then placed in a new 2 ml collection tube and centrifuged at 12,000 x g for 5 minutes. The RNeasy MinElute spin column was then placed in a

1.5 ml microcentrifuge tube and 14  $\mu$ l of RNase-free water was added to the column membrane and centrifuged for 1 minute at 12,000 x *g*. The flow-through was saved.

### **RT-PCR of *cLac* from Total RNA**

The Qiagen OneStep RT-PCR Kit and Protocol (Qiagen Inc.; Valencia, CA) was used for the reverse transcription of messenger RNA from the isolated total RNA and amplification of *cLac* using Polymerase Chain Reaction (PCR). The Qiagen OneStep RT-PCR Kit included dNTP Mix, 5X Qiagen OneStep RT-PCR Buffer, Qiagen OneStep RT-PCR Enzyme Mix, and RNase-free water. The template RNA was obtained through the isolation of total RNA from canine lacrimal tissue and *cLac*-Forward and *cLac*-Reverse primers were designed to incorporate an *Nde*I restriction site at the 5' end and a 3' *Sma*I site into the amplified product excluding the predicted protein signaling sequence, using the predicted sequence information from the genomic search tool Ensembl (Table 1). These primers were resuspended at 100  $\mu$ M concentrations and diluted to 6  $\mu$ M working solutions. Fifty microliter reactions were created within PCR tubes containing 10  $\mu$ l of Qiagen OneStep RT-PCR Buffer, 2  $\mu$ l of dNTP mix, 5  $\mu$ l of 6  $\mu$ M *cLac*-F primer, 5  $\mu$ l of 6  $\mu$ M *cLac*-R primer, and 2  $\mu$ l of Qiagen OneStep RT-PCR Enzyme Mix along with RNase-free water and DNA. The amounts of RNase-free water varied corresponded to the amount of template DNA used, having 2  $\mu$ l, 5  $\mu$ l, or 10  $\mu$ l of template DNA per reaction to reach a final volume of 50  $\mu$ l. The thermal cycler was programmed according to the protocol outlined in Table 2 and after completion of the RT-PCR reaction the samples were held at 4°C overnight and then visualized on a 1.5% agarose gel.

**Table 1.** Oligonucleotides used for PCR amplification of cLac gene.

Designation	Oligonucleotide sequence
cLac-F	5' GGA TTC <u>CAT ATG</u> GAA GGT GAC TCC TCA GAT CCT G 3' <i>NdeI</i> <i>cLac</i> →
cLac-R	5' GGT CCC <u>CCG GG</u> A TTG AAT TGT GGA ACA AGC TTT TTC 3' <i>SmaI</i> <i>cLac</i> →

**Table 2.** Cycling conditions for one-step RT-PCR using Qiagen OneStep RT-PCR Kit.

Step	Time	Temperature
Reverse Transcription	30 minutes	50°C
Initial PCR Activation	15 minutes	95°C
3-step Cycling	30 cycles	
Denaturation	30 seconds	94°C
Annealing	45 seconds	56°C
Extension	1 minute	72°C
Final Extension	10 minutes	72°C

### Vector pTYB2 Isolation

The Zyppy<sup>TM</sup> Plasmid Miniprep Kit was used to isolate pTYB2 from *E. coli* culture (Zymo Research; Irvine, CA). An *E. coli* culture containing the vector plasmid pTYB2 was grown in 50 ml LB broth overnight at 37°C in a shaking incubator. Six hundred microliters of *E. coli* was placed into a 1.5 ml microcentrifuge tube and 100 µl of 7X Lysis Buffer was mixed into the solution by inversion. Three hundred and fifty microliters of cold Neutralization Buffer was added to this solution and then centrifuged at 16,000 x g for two minutes. The supernatant was transferred into the Zymo-Spin IIN column placed within a collection tube and centrifuged for 15 seconds. The flow-through was discarded and 200 µl of Endo-Wash Buffer was added to the column and centrifuged for 15 seconds. Four hundred microliters of Zyppy Wash Buffer was added to the column and centrifuged for 30 seconds. The column was then transferred into a

clean 1.5 ml microcentrifuge tube and 30  $\mu$ l of Zyppy Elution Buffer was added directly to the column. The flow-through was collected and visualized with test enzymatic cuts with *NdeI* and *EcoRI* using a 1% agarose gel.

### Construction of *cLac* Intein Expression Plasmid

The *cLac* gene amplified from the RT-PCR underwent further amplification using PCR. Twenty-five  $\mu$ l of *Taq* 2X Master Mix (NE BioLabs; Ipswich, MA), 3  $\mu$ l of the RT-PCR reaction sample, 3  $\mu$ l of the *cLac*-F and *cLac*-R primers, and 16  $\mu$ l of distilled water were mixed and underwent PCR protocol outlined in Table 3. This PCR product was cleaned using the DNA clean and concentrator kit and protocol (Zymo Research; Irvine, CA) and visualized on a 1.5% agarose gel.

**Table 3.** Cycling conditions for PCR using NE BioLabs *Taq* 2X Master Mix.

Step	Time	Temperature
Initial Denaturation	5 minutes	95°C
3-step Cycling	30 cycles	
Denaturation	30 seconds	95°C
Annealing	30 seconds	56°C
Extension	45 seconds	68°C
Hold	Indefinitely	4°C

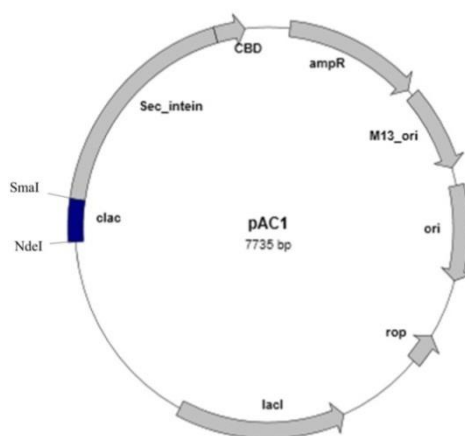
Amplified *cLac* gene samples and the isolated pTYB2 vector underwent restriction enzyme digest with *NdeI* and *XmaI*. Thirty microliters of each sample was combined with 1  $\mu$ l of *NdeI*, 1  $\mu$ l of *XmaI*, 5  $\mu$ l of NEB Buffer 4, and 13  $\mu$ l of distilled water and was incubated overnight at 37°C, cleaned, and then visualized on a 1.5% agarose gel.

The cut and cleaned *cLac* gene and pTYB2 vector were then ligated and transformed. Eight microliters of cut and cleaned pTYB2, 9  $\mu$ l of cut and cleaned *cLac* gene, 1  $\mu$ l T4 DNA Ligase, and 2  $\mu$ l of T4 DNA Ligase Reaction Buffer, 10X (NE BioLabs; Ipswich, MA) were



combined and incubated at 25°C for 20 minutes. The 20 µl were added to thawed competent *E. coli* strain ER2566 cells (NE BioLabs; Ipswich, MA) and incubated on ice for 30 minutes. The mixture was then heat shocked for 30 seconds at 42°C and then iced for 1 minute before 300 µl of LB broth was added to the mixture. This mixture shook for 1 hour at 37°C and 100 µl was plated onto a LB plate supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C.

The candidate *E. coli* colonies that grew on the LB Ampicillin plate were screened for the plasmid, pTYB2 containing the *cLac* gene, pAC1 (Figure 4) using PCR. Twenty colonies were selected and resuspended into 20 µl of distilled water. Eleven microliters was removed and placed into a 5 ml LB culture supplemented with 100 µg/ml ampicillin. The remaining nine microliters was incubated at 100°C for 5 minutes. Ten microliters of *Taq* 2X Master Mix (NE BioLabs; Ipswich, MA) and 0.5 µl of the cLac-F and cLac-R primers were added to the incubated samples and underwent PCR protocol outlined in Table 3. These PCR products were visualized on a 1.5% agarose gel (Figure 5). Two screened candidates were randomly selected, sequenced using universal forward T7 and reverse intien primers, and stored in 50% glycerol stocks at -60°C.



**Figure 4.** A schematic drawing of pAC1, the pTYB2-cLac intein expression plasmid.

## **Gene and Protein Alignment**

The DNA sequencing results for the cloned canine *cLac* gene was compared to the DNA sequence of the human *LACRT* gene examined previously. The DNA comparisons were performed using Clustal W2 alignment. The DNA sequences for both the canine *cLac* gene and the human *LACRT* gene were translated using the web ExPASy translate tool. The protein sequences were compared between the determined recombinant canine and recombinant human proteins using Clustal W2 alignment.

## **Agarose DNA Gel Electrophoresis**

Ethidium bromide staining of Agarose DNA Gel Electrophoresis was used to visualize separated DNA samples by size. DNA samples were prepared by mixing 7.5 ul of sample with 1.5 ul of loading buffer with dye. The agarose gel was poured by mixing agarose powder, at the desired (W/V) concentration, with 50 ml of Tris-borate-EDTA buffer (TBE) and heating this solution until the agarose powder has completely melted. A small amount of ethidium bromide, reaching a final concentration of approximately 0.5 µg/ml, was added to the heated solution and allowed to briefly cool. This solution was then poured into a casting tray that contains a comb and is allowed to solidify at room temperature for 1 hour. The comb was removed and the casting tray was inserted into the electrophoresis chamber with the wells near the negative electrode. The agarose gel was then completely covered in TBE and the prepared samples were loaded into each well along with the appropriate 100 bp or 1 kb DNA ladder (NE Biolabs; Ipswich, MA). The gel box was then assembled and ran at 150 V until the dye reaches near the end of the gel.

### **Purification of Recombinant cLac Protein from Intein-Fusion**

The cLac protein was purified using a chitin affinity column and ion exchange chromatography using DEAE Sepharose (NE Biolabs; Ipswich, MA). Ten milliliters of a 50 ml saturated overnight LB broth supplemented with 100 µg/ml ampicillin of the *E. coli* strain ER2566 containing pAC1 was used to inoculate 1 L of LB broth supplemented with 100 µg/ml ampicillin. The 1 L culture was shook at 37°C and induced with 1 ml of 0.5M IPTG when the OD<sub>600</sub> was between 0.5-0.7. The culture shook for 3.5 hours at 25°C and was then centrifuged at 6,000 rpm for 15 minutes. The supernatant was discarded and the pellet stored at -60°C.

Four one liter pellets were resuspended using 100 ml of lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, and 0.45% Triton X-100) and placed on ice. The suspension was sonicated for 75 seconds in 15 second bursts at 95% amplitude and then centrifuged at 13,000 rpm for 15 minutes saving the supernatant. A ten milliliter chitin column was equilibrated with ten column volumes of column buffer (50 mM Tris, pH 8.0, 500 mM NaCl). The supernatant collected after centrifugation of the lysed pellet was then run over the chitin column at a drip rate of 1 ml per 1 minute. The column was then washed with twenty column volumes of column buffer at a drop rate of 3 ml per 1 minute. Fifty milliliters of cleavage buffer (50 mM Tris, pH 8.0, 500 mM NaCl, and 0.40% B-Me-OH) was poured onto the column and 20 ml was allowed to drip at a rate greater than 5 ml per 1 minute stopping the column, leaving the cleavage buffer incubating on the column for more than 18 hours. Twenty to thirty milliliters of column buffer was added and 50 ml total of elution was collected from the column.

The fifty milliliters of elution was then concentrated to 4 ml using a 3 kD Amicon EMD Millipore Ultrafiltration Membrane (EMD Millipore; Billerica, MA). The concentrate was then dialyzed in 14 mM NaCl phosphate buffered saline (PBS) and 300 µl removed for assays. A two

milliliter DEAE Sepharose column was equilibrated with ten column volumes of 14 mM NaCl PBS. The dialyzed concentrate was then loaded onto the column and the flow-through collected. The column was washed with ten column volumes of 14 mM NaCl PBS, collecting 1 ml fractions. The column was then eluted with 140 mM NaCl PBS collecting 1 ml fractions. A final cut of 500mM NaCl PBS was also performed and 1 ml fractions collected.

### **BCA Total Protein Assay**

The Thermo Scientific Pierce<sup>TM</sup> BCA Protein Assay Kit and Protocol was used to determine the amount of total protein in each column chromatography fraction (Pierce Biotechnology; Rockford, IL). The working reagent for this assay, a solution containing bicinchoninic acid, was mixed using a 50:1 ratio of BCA Reagent A with BCA Reagent B. Two hundred microliters of the mixed working reagent was added to each individual well of a low binding 96-well microtiter plate. Twenty-five microliters of each tested sample was aliquoted into these wells. A standard curve ranging from 2,000-25 µg/ml was generated with bovine serum albumin (BSA) using the included dilution sets of the Albumin Standard Ampule, 2 mg/mL. The nine standards were aliquoted in duplicate across columns 1 through 9 of rows A and B, and the unknown fractions were aliquoted in duplicate in rows C and D of the microtiter plate (Appendix B). The microtiter plate was incubated at 37°C for 30 minutes and then the absorbance values the reactions were recorded at 570 nm using a spectrophotometer. The absorbance values recorded for the standard curve samples were graphed against the known concentrations of BSA and the line of best-fit equation for this standard curve was used to determine the total protein concentration for the unknown column chromatography fraction samples.

## **Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**

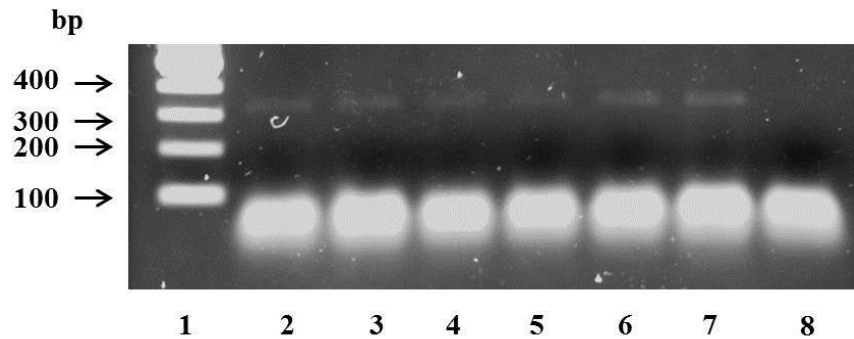
Coomassie brilliant blue staining of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to visualize separated proteins samples by size. Electrophoresis was performed prior to Coomassie brilliant blue staining of the polyacrylamide gel. The samples to be run were prepared using a 4:1 ratio of the column chromatography fraction samples and 5X SDS dye and boiled for ten minutes. The Bio-Rad minigel apparatus was assembled containing a Bio-Rad Any kD™ Mini-PROTEAN® TGX™ Precast Gel and the apparatus was filled with 1X Tris-glycine buffer. Seven microliters of Precision Plus Protein Kaleidoscope Standards maker and 25 µl of boiled sample were loaded into the designated lanes of the gel (Bio-Rad Laboratories; Hercules, CA). After loading the gel was run at 200 V until the markers reached near the bottom of the gel. The gel was then removed from the apparatus and rinsed with distilled water.

Coomassie blue stain was made using PlusOne Coomassie tablets PhastGel Blue R-350 (Ge Healthcare Bio-Sciences; Piscataway, NJ). One tablet was dissolved in 80 ml of distilled water and then 120 ml of methanol was added to the solution, which was then filtered for use. The gel was stained for 30 minutes in a one part coomassie blue stain and one part 20% acetic acid solution. The gel was then de-stained twice for 30 minutes in a 50% methanol and 5% acetic acid solution. The gel was then transferred to water and imaged using the Gel Doc™ XR+ System (Bio-Rad Laboratories; Hercules, CA).

## Results

### RT-PCR of *cLac* from Total RNA

Canine lacrimal tissue was obtained from Virginia Polytechnic Institute. This tissue was stored in *RNAlater* and total RNA isolation was performed on two, 3 mm cubes of the lacrimal tissue. Directly after isolation, this RNA solution underwent reverse transcription polymerase chain reaction (RT-PCR). The RT-PCR reaction was performed for both RNA samples with either 2  $\mu$ l, 5  $\mu$ l, or 10  $\mu$ l of RNA template per reaction, along with a no template control and results visualized on an agarose DNA gel (Figure 5). There were two distinct bands, one less than 100 bp in size and one approximately 325 bp in size, for each lane containing RNA template. There was one distinct band, less than 100 bp in size, for the lane containing no RNA template. The band less than 100 bp in size is a suspected primer dimer, while the band approximately 325 bp is the expected size of the canine lacritin gene.

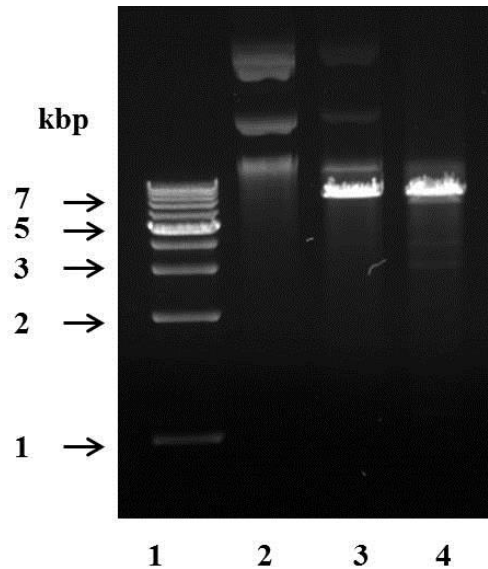


**Figure 5. Agarose DNA gel of RT-PCR samples of the total RNA extracted from canine lacrimal glands.** Lane 1 contains the 100 bp DNA ladder, lanes 2 – 4 contain one lacrimal gland sample with 2  $\mu$ l, 5  $\mu$ l, and 10  $\mu$ l of template DNA per reaction, lanes 5 – 7 contain one lacrimal gland samples with 2  $\mu$ l, 5  $\mu$ l, and 10  $\mu$ l of template DNA per reaction, and lane 8 contains no template DNA sample.

### Vector pTYB2 Isolation

An *E. coli* culture containing the plasmid pTYB2 was harvested using a plasmid mini-prep kit. Two separate enzymatic cuts were performed with restriction endonucleases *Nde*I and

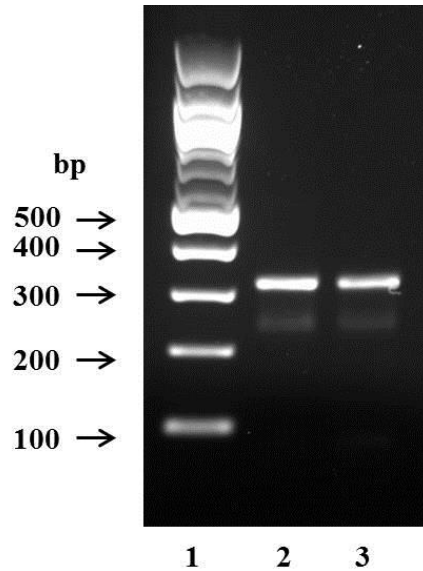
*EcoRI*. The cut products along with the uncut sample were visualized on an agarose DNA gel (Figure 6). The lane containing uncut pTYB2 has three different bands all running larger than the kbp ladder used. The lanes containing the restriction enzyme cuts each contain a strong bright band that is greater than 7 kbp.



**Figure 6. Agarose DNA gel of the isolated pTYB2 vector.** Lane 1 contains the 1 kbp DNA ladder, lane 2 contains uncut pTYB2, lane 3 contains pTYB2 cut with *EcoRI*, and lane 4 contains pTYB2 cut with *NdeI*.

### Construction of *cLac* Intein Expression Plasmid

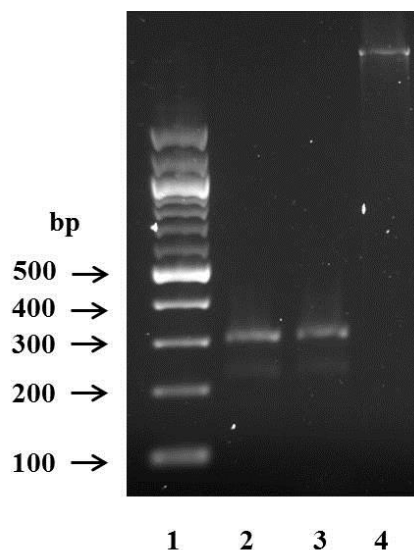
The RT-PCR products underwent further PCR amplification using the cLac-F and cLac-R primers. This PCR amplification was visualized on an agarose DNA gel for two samples (Figure 7). The lanes containing the PCR amplifications contain a strong band at about 325 bp in size and a faint band at about 250 bp in size.



**Figure 7. Agarose DNA gel of the RT-PCR product after further PCR amplification.** Lane 1 contains the 100 bp DNA ladder, lanes 2 and 3 contain the amplified products.

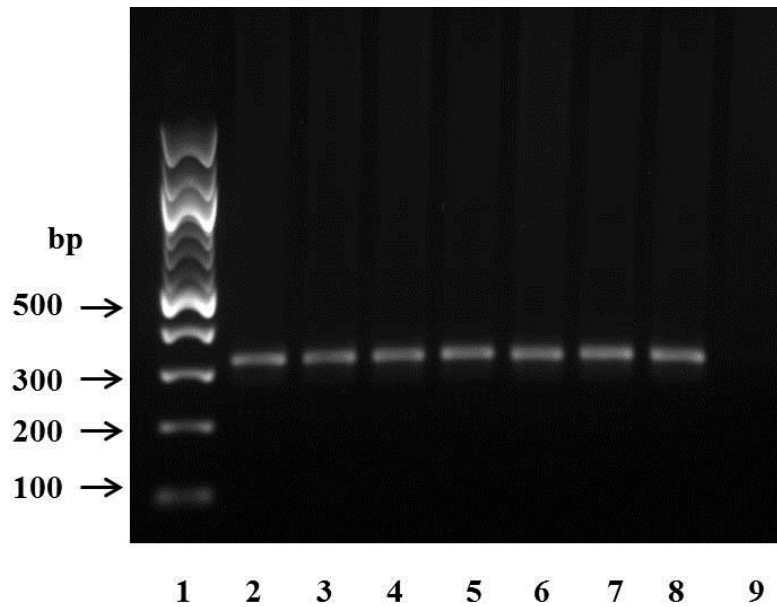
Restriction endonuclease digests were performed using *NdeI* and *XmaI* on the PCR products and the pTYB2 vector and visualized on an agarose DNA gel (Figure 8). The lanes containing the cut PCR amplification products have a distinct band at about 325 bp in size and also a very faint band at about 250 bp in size. The lane containing cut pTYB2 has a distinct band traveling much higher than the 100 bp DNA ladder.





**Figure 8. Agarose DNA gel of the cut pTYB2 vector and the amplified *cLac* gene with *Xma*I and *Nde*I.** Lane 1 contains the 100 bp DNA ladder, lanes 2 and 3 contain cut insert, and lane 4 contains cut pTYB2.

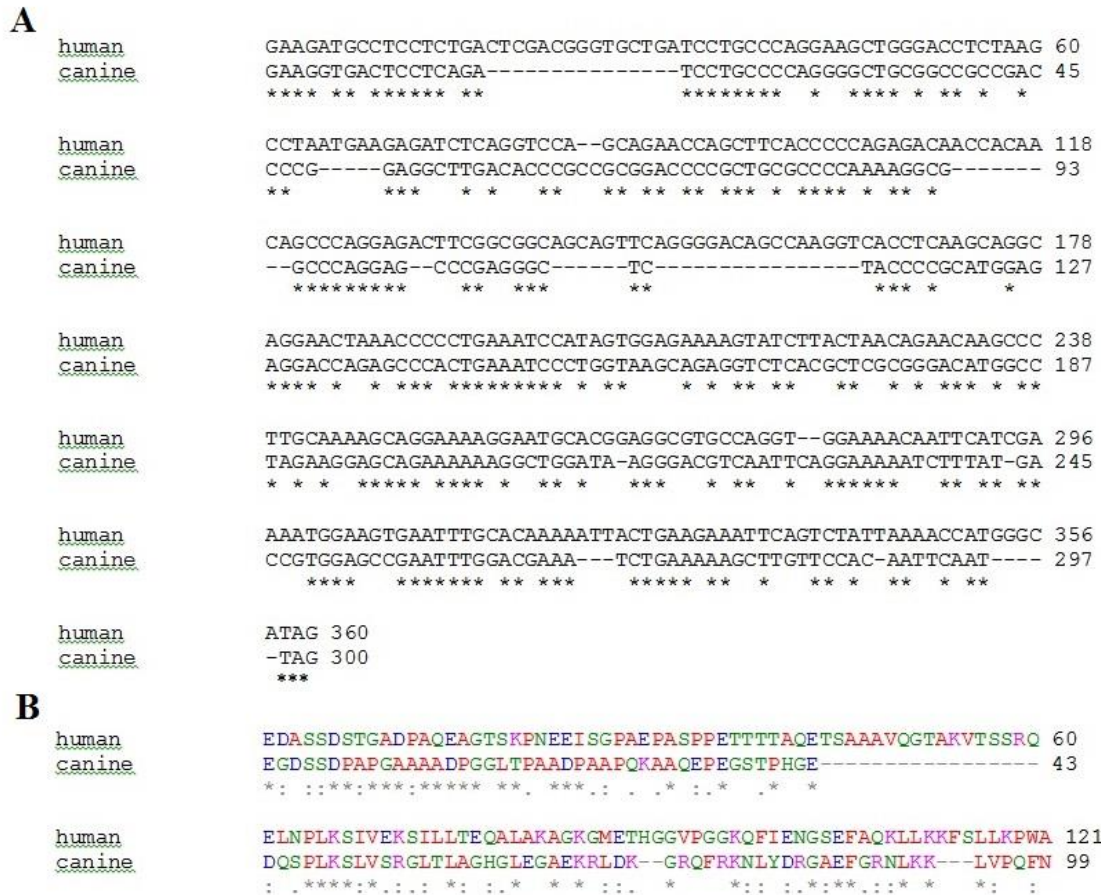
The candidate *E. coli* colonies that grew on the LB Ampicillin plate after ligation and transformation were screened for the plasmid pAC1 using PCR. The cLac-F and cLac-R primers were used for the PCR and the screening results were visualized on an agarose DNA gel (Figure 9). Lanes 2 – 9 contain the PCR products of eight screened candidates. Lanes 2 – 8 contain a distinct band at 325 bp in size, while lane 9 does not contain a distinct band.



**Figure 9.** Agarose DNA gel of the transformant candidate screening Lane 1 contains the 100 bp DNA ladder, lanes 2 – 9 contain the PCR product of eight screened candidates.

### Gene and Protein Alignment

The sequenced canine *cLac* gene was aligned and analyzed against the human *LACRT* gene using Clustal W2 analysis (Figure 10). The analysis reveals that the cloned *cLac* gene is 300 nucleotides in length, compared to the cloned human *LACRT* gene of 360 nucleotides in length. The recombinant canine lacritin, cLac, amino acid sequence has a 29% identity to recombinant human lacritin.

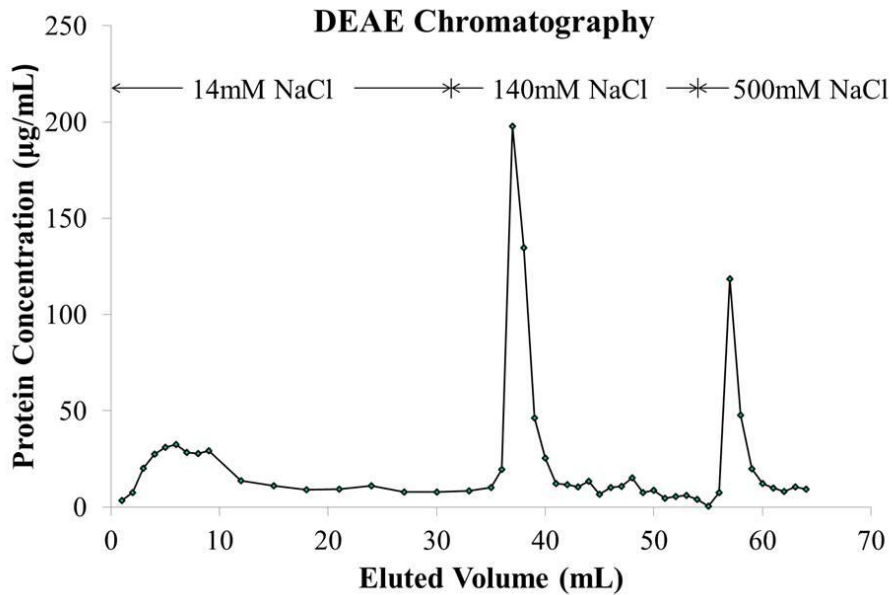


**Figure 10. Gene and Sequence Alignment of the cloned canine lacritin sequence to cloned human lacritin.** (A) The nucleotide alignment for the cloned human lacritin gene with the sequenced cloned canine *cLac* gene. (B) The protein alignment for recombinant human lacritin and the recombinant canine lacritin.

**Purification of Recombinant cLac Protein from Intein-Fusion**

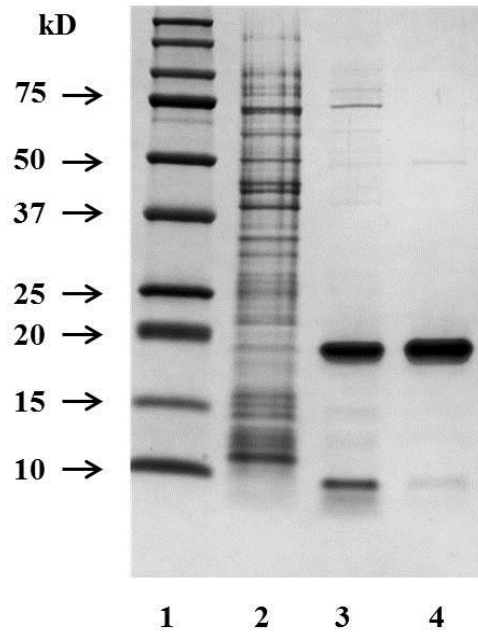
The cLac protein was purified using affinity and ion exchange chromatography. Four liters of induced cleared lysate was run over a chitin column, cleaved, eluted, and dialyzed in 14 mM NaCl PBS. The dialyzed elution was run over DEAE Sepharose and the flow through and wash were collected. 140 mM NaCl PBS and 500 mM NaCl PBS cuts were then eluted from the DEAE sepharose. The fractions collected from the DEAE sepharose were assayed for total protein concentration to produce a salt elution profile (Figure 11). There was a slight peak from

3 – 10 mL of eluted volume, a large peak at 35 – 38 mL of eluted volume, and a peak at 55 – 58 mL of eluted volume.



**Figure 11. 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.

The cleared lysate, the dialyzed chitin elution, and the peak from the 140 mM NaCl PBS cut were visualized using SDS PAGE (Figure 12). The cleared lysate contained a smear of proteins along the gel. The dialyzed chitin elution contains a strong band around 18 kD in size, a strong band below 10 kD in size, and other bands along the gel. The peak from the 140 mM NaCl PBS cut has a strong band at 18 kD in size, and a couple bands along the gel.



**Figure 12. SDS PAGE Gel of 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 140mM NaCl PBS elution from the DEAE Sepharose column.

## Discussion

Corneal health and function is maintained through the presence of tears (Ofri, *et. al.*, 2009). A deficiency in tear production has been observed throughout the human species (Moss, *et. al.*, 2000). Keratoconjunctivitis sicca (KCS) has been observed in 1.5% of canines visiting veterinary hospitals and clinics. Clinical drugs have been tested and developed to treat human KCS, and a current treatment for canine KCS is the human therapeutic RESTASIS<sup>®</sup> (Ofri, *et. al.*, 2009). Therefore, canine KCS may provide an acceptable model system for pre-clinical animal trial and the development of a canine therapeutic may provide a comparison study for a human therapeutic.

The human *LACRT* gene has been aligned to the canine chromosome 27, and Clustal W2 analysis revealed a potential canine lacritin ortholog with a 35% amino acid identity to human lacritin (Laurie, *et. al.*, 2012). The presence of a potential canine lacritin ortholog was examined using Western blot analysis and an indirect ELISA developed using polyclonal rabbit antibodies against the 19 amino acid human lacritin N-terminus and the N-65 truncation mutant of human lacritin. Preliminary research with canine tear samples detected the presence of a canine lacritin-like protein using the human indirect ELISA and Western blot analysis protocols.

The detection of a canine lacritin-like protein was achieved with one of the two human polyclonal antibodies. This observation, along with the knowledge that the proteins were only 35% similar led to further exploration of this canine lacritin-like protein. Using the preliminary chromosome and sequence alignments, a cloning strategy was developed to isolate, purify, and study canine lacritin in further detail. Sequencing and purifying the canine lacritin protein will assist in the development of clinical assays for a pre-clinical canine clinical study.

The cloning strategy encompassed primer design and isolation of mature messenger RNA from canine lacrimal gland tissue. Using the predicted canine lacritin gene sequence, primers were designed to amplify the canine lacritin gene. The primer design excluded the leader sequence of the canine lacritin gene to facilitate the production of recombinant canine lacritin in the processed biological state. The primer design also incorporated restriction endonuclease cut-sites for *NdeI* and *SmaI* to facilitate cloning the amplified gene into the pTYB2 intein expression system (Table 1).

Canine lacrimal gland tissue stored in *RNAlater*, to preserve RNA from degradation, was obtained from Virginia Polytechnic Institute. The lacrimal tissue was then homogenized and the total RNA was extracted from the tissue. To avoid RNA degradation, the total RNA sample underwent reverse transcription polymerase chain reaction (RT-PCR). The RT exploited the enzyme reverse transcriptase to transcribe only mature messenger RNA into cDNA. The PCR amplification used the specifically designed primers for the canine lacritin gene to produce an amplified copy of the mature canine lacritin gene. The predicted canine sequence was 369 nucleotides long; however, the 57 nucleotides associated with the leader sequence were excluded in the amplification. An amplified PCR product was visible on an agarose gel at approximately 325 nucleotides in size corresponding to the predicted size of the targeted canine lacritin gene, with a strong band less than 100 nucleotides in size resulting from primer dimers expected with the high concentration of primers used in the reaction.

The developed cloning strategy exploits cloning the gene of interest into the pTYB2 *E. coli* cloning and expression vector (NE BioLabs; Ipswich, MA). The pTYB2 vector contains a multiple cloning site with *NdeI* and *SmaI* cut-sites upstream of the fusion protein intein, along with ampicillin resistance. The pTYB2 vector was harvested from an *E. coli* culture to use in the

cloning procedure. Restriction enzymes were used to cut the harvest pTYB2 to test the purity and viability of the vector. Two separate test cuts were performed using *NdeI* and *EcoRI* and viewed on an agarose gel (Figure 6). The uncut vector contained both supercoiled and nicked pTYB2 vector viewable. The two separate test cuts contained a bright band larger than 7,000 nucleotides in length. After isolating the gene of interest, the insert, and the pTYB2 vector the construction of the *cLac* intein expression plasmid began.

The construction of the *cLac* intein expression plasmid included enzymatic digestion, ligation, transformation, and candidacy screening. Prior to enzymatic digestion, the RT-PCR product underwent PCR amplification to obtain a higher concentration of the insert canine *cLac* gene. The PCR product and pTYB2 vector both underwent enzymatic digestion using *NdeI* and *XmaI* (Figure 8). The restriction enzyme *XmaI*, and isoschizomers of *SmaI* was used to create a second sticky end and to use an enzyme of similar active conditions as *NdeI*. The sizes of both the cut *cLac* gene and pTYB2 vector were as predicted, and their concentrations were estimated based upon band intensity for the ligation reaction. The vector and insert were ligated and transformed into *E. coli*. The *E. coli* was plated onto LB ampicillin plates to select for *E. coli* that up-took the vector. Further screening of the potential candidates was necessary, because the ampicillin resistance would be present regardless of the insertion of the *cLac* gene into the vector. To screen the potential candidates, the vectors underwent PCR using the primers designed specifically for the *cLac* gene (Figure 9). The candidates containing only pTYB2 would not have an amplified production after PCR, while candidates containing pAC1 would have an amplified strand of DNA at approximately 325 nucleotides in length correlating to the *cLac* gene. Out of twenty selected candidates, thirteen contained pAC1. Two candidates were randomly selected and pAC1 harvested from these cultures was sent for DNA sequencing.



The cLac gene was compared to the predicted sequence from the genomic search tool Ensembl having a 100%, with the leader sequence removed. Clustal W2 analysis was used to compare the sequenced canine *cLac* gene and the human *LACRT* gene and their respective recombinant proteins (Figure 10). There was a 29% homology between the two amino acid sequences, with multiple similarities in the N-terminal region. These similarities in the N-terminal region may explain the detection of low amounts of canine lacritin using antibodies developed against human lacritin.

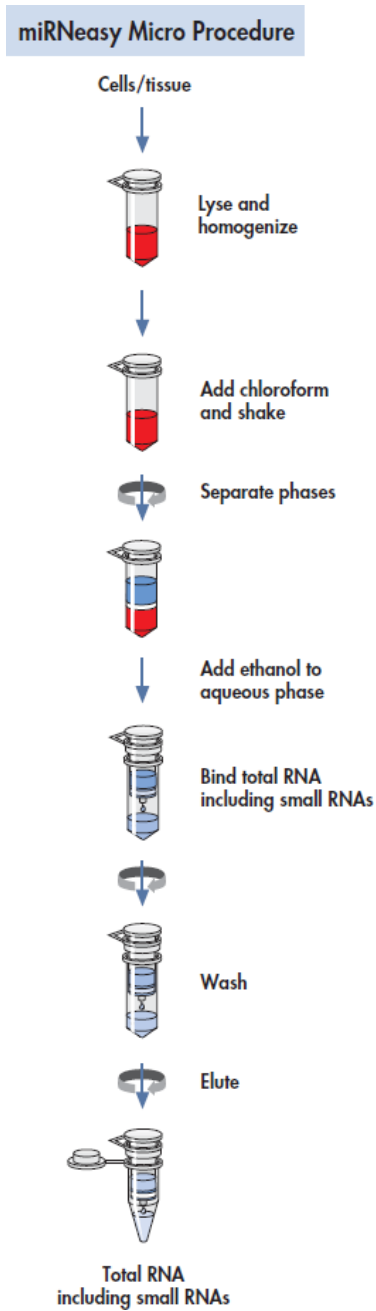
Purification of canine lacritin will assist in the development of canine specific assays and a potential canine therapeutic. Knowledge of the *cLac* intein expression plasmid assisted in the development of the purification strategy for the canine lacritin. Four liters of *E. coli* was induced for expression of the pAC1 lac operon, containing cLac fused to intein and the cleared lysate was purified using column chromatography (Figure 12). The fusion protein, intein, binds to chitin; therefore, the cleared lysate after induction was run over a chitin column. The cleavage buffer, containing B-Me-OH was used to facilitate on-column cleavage releasing the canine lacritin from the intein bound to the chitin. Elution from the column contained canine lacritin and a few various unidentified proteins. This elution was then dialyzed in 14 mM NaCl PBS to exploit ion exchange chromatography using DEAE sepharose, a strong cation exchanger. The elution was run over the DEAE sepharose and the flow through and washes were collected. The DEAE sepharose was then eluted with 140 mM NaCl PBS and 500 mM NaCl PBS salt cuts and fractions were collected. The total protein concentrations for each fraction were determined using a Pierce<sup>TM</sup> BCA Protein Assay Kit and graphed to produce an elution profile (Figure 11). Each peak observed in the elution profile represents the various proteins eluting from the DEAE sepharose dependent on the cation exchange occurring with each salt cut. SDS-PAGE reveals

that canine lacritin migrating between 15 and 20 kilo-Daltons was eluted with the 140 mM NaCl PBS salt cut with greater than 95% purity (Figure 12). This purified protein may be used for the development of canine specific assays along with a potential canine therapeutic for KCS.

The cloning and purification of canine lacritin is essential to the development of a potential pre-clinical study using a canine KCS model. The isolation of canine lacritin will allow for the development of canine specific assays to use in the visualization and quantification of canine lacritin in canine tear samples. This clinical data will facilitate the development of a study using a canine KCS model. Canine lacritin will be able to be tested for increased basal secretions in healthy canines, similar to that observed in healthy rabbits with the use of human lacritin (Samudre *et. al.*, 2011). These clinical findings may lead to the development and use of canine lacritin as a therapeutic in a canine KCS model. The cloning and purification of canine lacritin has opened further research opportunities in developing human lacritin as a human therapeutic for dry eye.

## Appendix A

### Qiagen miRNeasy Micro Kit Protocol



**Appendix A.** Visual protocol of the miRNeasy Micro Kit protocol using spin column chromatography. Image obtained from Qiagen Sample & Assays Technology miRNeasy Micro Handbook, Feb 2012.

## Appendix B

### Diagram of BCA Protein Assay Microtiter Plate

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>
<b>A</b>	A	B	C	D	E	F	G	H	I
<b>B</b>	A	B	C	D	E	F	G	H	I
<b>C</b>	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9
<b>D</b>	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8	Fraction 9

**Appendix B.** Diagram of BCA Protein Assay microtiter plate. The BSA standards were added in duplicate to columns 1 to 9 of rows A and B, while the unknown fraction samples were added in duplicate to rows C and D.

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