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Levels of human tear lacritin isoforms in healthy adults

Brooke Justis

Levels of Human Tear Lacritin Isoforms in Healthy Adults

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FACULTY COMMITTEE:

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

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

Reader: Louise Temple, Ph.D.
Professor, ISAT

HONORS COLLEGE APPROVAL:

Bradley R. Newcomer, Ph.D.,
Dean, Honors College

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ABSTRACT

Current diagnostics of dry eye disease are unable to identify the multiple complex causes of dry eye, thereby limiting the proper treatments of the disease. The tear glycoprotein lacritin has been reported to be decreased in dry eye patients, specifically those with Sjögren's syndrome associated dry eye. Therefore, the use of lacritin in a diagnostic test for dry eye could improve the current diagnostics available. Western blot assays have revealed the presence of an active lacritin monomer as well as an inactive tissue transglutaminase cross-linked polymer in human tears. A third isoform of lacritin, the splice variant lacritin-c, has also been detected in human tears. Using a diagnostic multiplex western blot assay, the three lacritin isoforms were quantitated in forty samples of human tears from twenty adults and analyzed using JMP software. This provided a baseline distribution of percent lacritin in human tears that will be used in future lacritin clinical trials.

INTRODUCTION

One of the most common problems in ophthalmology is dry eye disease; however, diagnosis is difficult due to multiple causes of the disease and lack of a definitive diagnostic test. Currently, there is no consensus on the guidelines for diagnosis, and the causes of dry eye are complex, ranging from premenopausal symptoms in women to altered corneal innervation (McNamara et al., 2016). Dry eye consists of a wide spectrum of dysfunctions and there is no specific separation between an eye designated as "normal" and an eye that presents dry eye. Additionally, the current diagnostic tests for dry eye are not standardized, making comparison between patients difficult (Savini, 2008). The most commonly used diagnostic tool for dry eye diagnosis is the Schirmer tear test (STT) which measures total tear secretion by inserting Schirmer tear test strips into the temporal lower conjunctive sac to measure the rate of tear

production (Boyd, 2018). After five minutes, the strip is removed, and wetting is recorded in millimeters (mm). Normal tears are diagnosed by wetting over 10mm, and dry eye tears are diagnosed by wetting less than 10mm. Unfortunately, there are multiple issues with using the Schirmer tear test that include low reproducibility, sensitivity, frequent discomfort, difficulty performing tests in children, and potential injury to conjunctiva and cornea (Salomon-Ben Zeev et al., 2014). Due to varying diagnostics and causes for dry eye there is a need for a reproducible test for the disease.

One of the emerging technologies for the diagnosis of dry eye is the use of tear biomarkers to analyze tear protein patterns and identify dry eye in a protein-specific manner. The tear glycoprotein, lacritin, has demonstrated potential as a protein biomarker for dry eye (Karnati et al., 2013). Lacritin is a prosecretory mitogenic protein that is known to promote basal tearing and exhibits cytoprotective activity (Wang et al., 2015). Structurally, lacritin has a molecular weight of 12.3 kDa, an isoelectric point of 5, and consists of 119 amino acids; tertiary structure of lacritin consists of two alpha helices, one amphipathic, and one that supports ligand-ligand binding (Ma et al., 2008). The lacritin gene contains 5 coding exons and 4 noncoding introns. The splice variant, lacritin-c, lacks exons 4 and 5 entirely and has sequences encoding 39 amino acids found in intron 3 (McKown et al., 2010). When cleaved, lacritin releases the amphipathic a-helical fragment, which creates bactericidal activity of tears by promoting metabolic stress and eventual bacterial death (McKown et al., 2014).

Lacritin promotes ocular health but is seen to be downregulated in dry eye and other dry eye related diseases. An example of a dry eye related disease with a connection to lacritin is Sjögren's syndrome, which has been linked to low levels of lacritin (McNamara et al., 2016). Sjögren's syndrome (SS) is an autoimmune disease in which dry eye is one of the main

symptoms, and currently there is no cure for this disease. However, when lacritin is present in sufficient amounts for healthy individuals, the protein restores corneal homeostasis and promotes basal tear secretion, as well as, epithelial cell proliferation (Karnati et al., 2016), (Wang et al., 2006). Therefore, lacritin demonstrates an inverse relationship to dry eye symptoms.

Because of the relationship between lacritin and dry eye, lacritin in ocular disease has been studied to determine the use of lacritin as a potential therapeutic. In a study comparing lacritin treatment in rabbits with cyclosporin and artificial tears, lacritin was found to increase basal tearing in rabbits, while the other treatments were unable to match the same results (Samudre et al., 2011). Another study utilized a dry-eye treatment consisting of thermo-responsive lacritin combined with an elastin-like polypeptide, which increased tear secretion in mice (Wang et al., 2015). Therefore, lacritin can promote natural tear secretion in dry eye when topically applied. Additionally, lacritin has demonstrated protective activities after corneal epithelia exposure to benzalkonium chloride (BAK), a preservative found in eye drops. BAK causes ocular surface inflammation and toxicity, but when lacritin is applied to corneal epithelia exposed to BAK, there is increased cell proliferation, suggesting the use of lacritin as a treatment for BAK exposure (Feng et al., 2014). This protective role of lacritin was also shown to alleviate apoptosis of lipopolysaccharide challenged corneal epithelial cells (Vantaku et al., 2016). These studies demonstrate lacritin's potential use as a biotherapeutic, as lacritin activity targets the causes of dry eye rather than treating the symptoms (Karnati et al., 2013).

Sjögren's syndrome, an autoimmune disorder characterized by severe dry eye, has been shown to have a strong relationship with lacritin. In a previous study, SS patients and normal controls were assayed for lacritin proteins by western blot analysis, which revealed that monomeric lacritin was downregulated in SS patients when compared to normal controls

(Vijmasi et al., 2014). In another study with SS patients, active tear lacritin was reduced in SS patients when compared to age matched controls, and reduced levels of lacritin were correlated to dry eye symptoms in SS patients (McNamara et al., 2016). Thus, the relationship between SS associated dry eye and lacritin suggests that the protein has potential as both a diagnostic biomarker and a topical therapeutic for SS dry eye.

Herein is reported the application of a multiplex western blot assay to quantify tear lacritin isoforms in twenty healthy adults. These isoforms consist of monomeric lacritin, the splice variant lacritin-c, and a cross-linked form of lacritin formed from an inactive tissue transglutaminase cross-linked polymer, which negatively regulates lacritin activity (Velez V et al., 2013). Tear samples from twenty healthy adults were eluted and analyzed by multiplex western blots. This was performed in preparation for receipt of samples from the phase 2 trial of Lacripep™ in Sjögren's syndrome Dry Eye and from a non-interventional study of lacritin from healthy volunteers. Samples from both the right (oculus dexter-OD) and left (oculus sinister-OS) eyes were collected from both male and female subjects of varying ages. By quantitating different forms of lacritin among twenty healthy subjects, we can provide a baseline for future analysis of tear lacritin proteins in diseases such as Sjögren's syndrome.

MATERIALS AND METHODS

Collection and Elution of Tears

Normal human tears were collected using Schirmer tear test strips that were placed in cryotubes and stored at -20° C at the University of California San Francisco (UCSF). All activities followed the guidelines set by the Declaration of Helsinki and approval by the UCSF

Committee for Human Research, as well as HIPPA laws. Informed consent was obtained from all subjects.

Once the tear samples were collected, the Schirmer strips were shipped on dry ice and stored at -60°C until thawed for elution. For elution, sixty microliters of phosphate buffer saline (PBS) were loaded onto the thawed Schirmer strips placed in spin baskets inserted into microcentrifuge tubes. Tubes were incubated for twenty minutes at room temperature and then centrifuged at 13,000 rotations per minute (RPM) for an additional ten minutes at 4°C. The eluted products were analyzed with the Bicinchoninic Acid Assay (BCA) for determination of total protein concentration (µg/mL) eluted from each tear sample.

Bicinchoninic Acid Assay (BCA)

The Thermo Scientific Pierce BCA Protein Assay Kit was utilized to determine total protein concentration of each tear sample against a protein standard. Tear samples were loaded in duplicate into a 96 well microtiter plate along with bovine serum albumin (BSA) standards ranging from 0 mg/mL to 2.0 mg/mL. Samples and standards were loaded in volumes of 10uL and analyzed at 570 nm using a spectrophotometer, following a thirty-minute reaction with working reagent at 37°C. Unknown concentrations of total protein in each tear sample were identified in mg/mL after comparison with absorbance values and known concentrations generated through a BSA standard curve.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blots

Tear samples were normalized to 400 µg/mL and loaded onto Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) and electrophoresed at 200 volts via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). For assay

validation, 40 tear samples were divided into 5 sets (101-104, 105-107&109, 110-113, 108&114-116, 117-120 OD, OS) and each set was run in duplicate. Each gel was transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany) using a trans-blot apparatus run at 100 volts for one hour (Appendix B). Blots were stored at 4°C until probed with antibodies.

For development, the blots were washed for ten minutes with phosphate buffer saline and 0.3% tween-20 (PBS-T) for a total of four washes at ten minutes each. Blots were incubated with either anti-Lacritin N-term rabbit polyclonal antibodies (1:1500 dilution in PBS-T) or anti-Lacritin I3 rabbit polyclonal antibodies (1:1500 dilution in PBS-T), developed from Bio-Synthesis Inc (Lewisville, TX). N-term antibodies recognize all three lacritin isoforms, while I3 antibodies recognize only the splice variant and cross-linked isoforms of lacritin. The incubation took place for one hour at room temperature, and blots were rinsed with PBS-T for an additional four washes of ten minutes each, then incubated again for one hour with fluorescently labeled goat anti-rabbit antibodies (1:12,500 dilution in PBS-T), followed by four washes with PBS-T at 10 minutes each.

Imaging and Quantitation of Lacritin Isoforms

Blots were imaged on a LiCor Odyssey CLx imaging system. The Odyssey CLx utilizes two lasers that scan the blots twice, once using each laser's high wavelength (800nm), and another using each laser's low wavelength (700nm). These scans from each laser come together to form one image (Bouzid, Ahmed, Lesiak, 2013). Using ImageSoftware Lite, quantitation of lacritin isoforms was determined based on relative signal obtained from the Odyssey CLx image. Using a standard curve generated from recombinant lacritin standards of known concentrations on each blot, nanograms (ng) of lacritin were calculated. From the nanograms of lacritin and the nanograms total protein, the percent of total lacritin and individual lacritin isoforms were also

calculated. The lacritin percentages among the samples were distributed using a quartile system obtained from JMP software.

RESULTS

Optimization of the Multiplex Western Blot Assay

In this study, the multiplex western blot assay was optimized to determine the most efficient antibody dilutions, as well as, concentrations of both recombinant lacritin standards and human tear samples to be used in the western blot. The LI-COR Odyssey CLx imaging system, used to detect the presence of each lacritin isoform, demonstrated that primary antibody dilutions of 1:1500 and secondary antibody dilutions of 1:12,500, as well as, loaded samples of 400µg/mL displayed the most clear and distinct western bands to be used in quantitation. This allowed for more precise quantitation of each lacritin isoform, providing accurate identification for the distribution of mass and percent lacritin isoforms throughout the forty samples.

Antibodies Detect Different Isoforms of Lacritin in Human Tears

To detect lacritin isoforms in the multiplex western blot assay, antibodies were produced that would identify the monomeric, cross-linked, and splice variant isoforms. The lacritin gene contains five coding exons separated by four introns which produces the mature monomeric protein of 119 amino acids found in human tears. The splice variant, lacritin-c contains exons 2 and 3 fused in-frame to 39 amino acids of intron 3, which produces a truncated protein of 104 amino acids also found in human tears (unpublished data). Rabbit polyclonal antibodies were produced against the first 22 amino acids of exon 2 (N-Term Antibody) and 31 amino acids of intron 3 (I3 Antibody) (Figure 1A).

One western blot of six tear samples and recombinant lacritin was challenged with anti-lacritin N-Term Antibody, and a second blot with the same samples was challenged with anti-

lacritin I3 Antibody (Figure 1B and 1C). Western blots developed by fluorescent secondary antibodies and imaged by the LI-COR Odyssey CLx imaging system revealed the anti-lacritin N-Term antibodies detected full length tear lacritin (monomer), recombinant lacritin, and lacritin-c (Figure 1B) while I3 antibodies only detected lacritin-c specific sequences (Figure 1C). Both antibodies detected cross-linked forms of lacritin (Figure 1B & 1C). These antibodies were then used to detect the lacritin isoforms from the twenty samples shipped from UCSF.

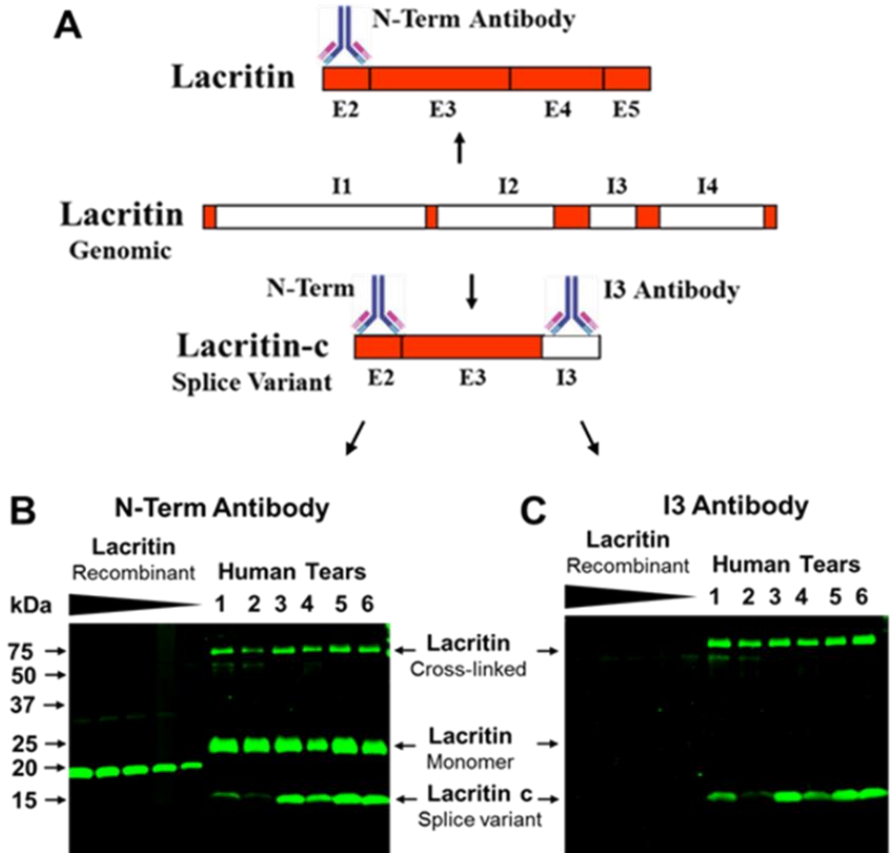


Figure 1. Antibodies Detect Different Isoforms of Lacritin in Human Tears. (A) Anti-lacritin antibodies were produced against N-term sequences and unique sequences of the splice variant lacritin-c. **(B)** N-term antibodies detect monomeric lacritin, cross-linked lacritin, lacritin-c in human tears and recombinant lacritin produced in bacteria by Western blot analysis. **(C)** Antibodies unique to intron 3 of lacritin (I3) detect only lacritin-c and cross-linked forms of lacritin-c by Western blot analysis.

Quantitation of Tear Lacritin Isoforms by Western Blot

To quantify the tear lacritin isoforms on western blots, known masses of purified lacritin (ng) were run along with tear samples on the same blot and used to generate a standard curve. A western blot containing six human tear samples and the purified recombinant lacritin standards was probed with anti-lacritin N-Term antibodies, developed with fluorescent secondary antibodies, and imaged by the LI-COR Odyssey CLx imaging system (Figure 2A). The LI-COR Odyssey CLx imaging system produced a signal number for each band identified on the blot generated by the near-infrared fluorescent detection of the used secondary antibodies (Figure 2A). Near-infrared fluorescent detection of purified recombinant lacritin standards were plotted against the known masses of those same standards to generate a standard curve of signal versus lacritin (ng) (Figure 2B). Specific protein bands for lacritin, lacritin-c, and cross-linked polymers of lacritin from human tears were quantified by near-infrared fluorescent signals, and a linear regression equation ($y=1.09x$) generated from recombinant lacritin was used to approximate the masses of tear lacritin proteins (ng) (Figure 2C)**.**

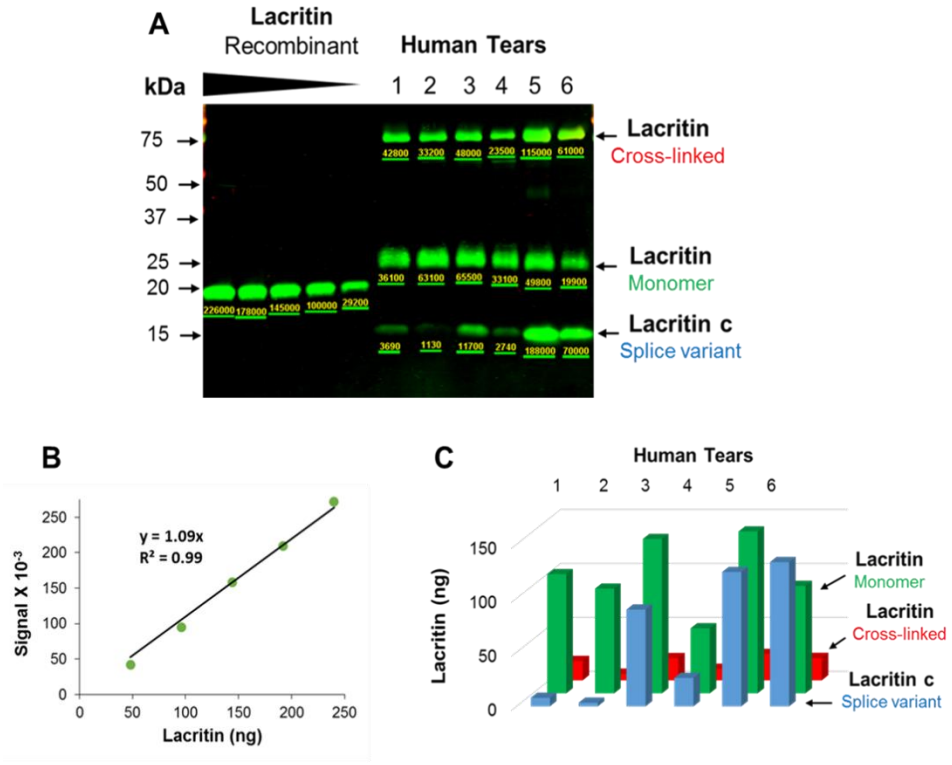


Figure 2. Western Blot Quantitative Analysis. (A) Human tear samples and recombinant lacritin challenged with anti-lacritin N-Term primary antibodies and fluorescent secondary antibodies **(B)** Decreasing concentrations of recombinant lacritin were quantified using near-infrared fluorescent detection and plotted against known masses of recombinant lacritin (ng) to generate a standard curve of signal versus lacritin (ng). **(C)** Specific protein bands for lacritin, lacritin-c, and cross-linked polymers of lacritin from human tears were quantified and a linear regression equation was used to approximate concentrations of tear lacritin proteins**.**

Assay Validation with Tear Samples from 20 Adults

In order to validate the immunoassay, tear samples from both eyes (OD and OS) of twenty adults were first analyzed for total eluted protein. Total protein concentration of the eluted samples was determined using the bicinchoninic acid assay (BCA) (Appendix A). The distribution of protein concentrations (Figure 3A) was divided into quartiles using JMP software (Figure 3B). The 25th quartile, median (50th) quartile, and 75th quartile total protein concentrations were 1.5025 mg/mL, 1.92 mg/mL, and 2.3175 mg/mL, respectively (Figure 3B).

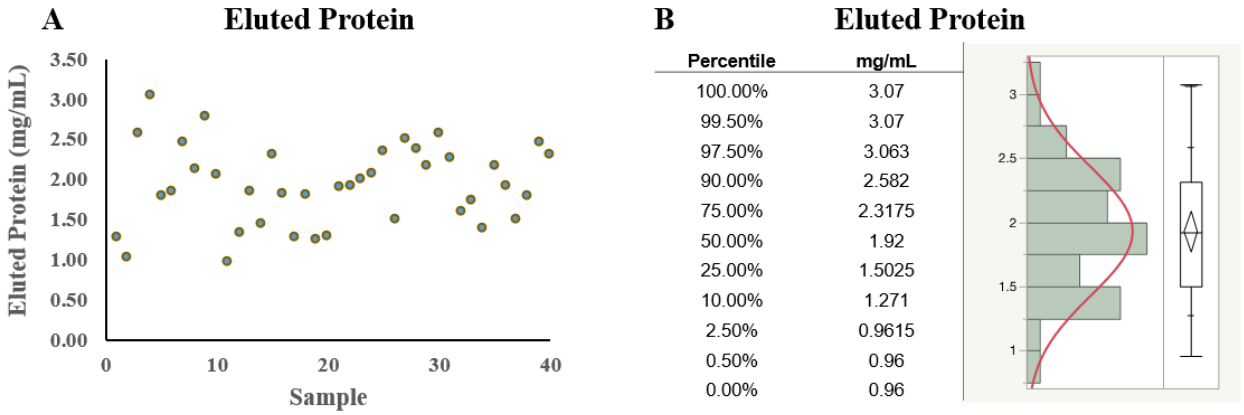


Figure 3. Distribution of Total Eluted Protein from 20 Adults (40 samples). Distribution of eluted protein concentrations versus sample number as a scatter plot **(A)** and the distribution of eluted protein based on a quartile system **(B).** The standard deviation is represented to the right of the curve along with tick marks denoting the 5th and 95th percentiles of the curve. Additionally, the curves are divided into quartiles (25th, 50th, and 75th) as denoted by the box to the right of the curve. The diamond within this quartile box represents the mean of the samples, the middle of the diamond is the mean and the tips of this diamond represent the certainty of the mean.

Western Blot Analysis of Tear Samples from 20 Adults

To separate and detect the lacritin isoforms, tear samples were loaded on Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad), electrophoresed at 200 V, and transferred to nitrocellulose (Protran BA 83; Whatman, Dassel, Germany). Blots were blocked with PBS + 0.3% Tween 20 (PBST), incubated with anti-Lacritin N-Term (1:500 dilution in PBST) for 1 hour at room temperature, rinsed with PBST, and incubated for 1 hour at room temperature with fluorescently labeled goat anti-rabbit antibodies (1:12,500 dilution in PBS-T). Western blots were imaged with a LI-COR Odyssey CLx imaging system which revealed the presence of each lacritin isoform: cross-linked lacritin (75kDa), monomeric lacritin (25kDa), and the lacritin splice-variant (15kDa) (Figure 4).

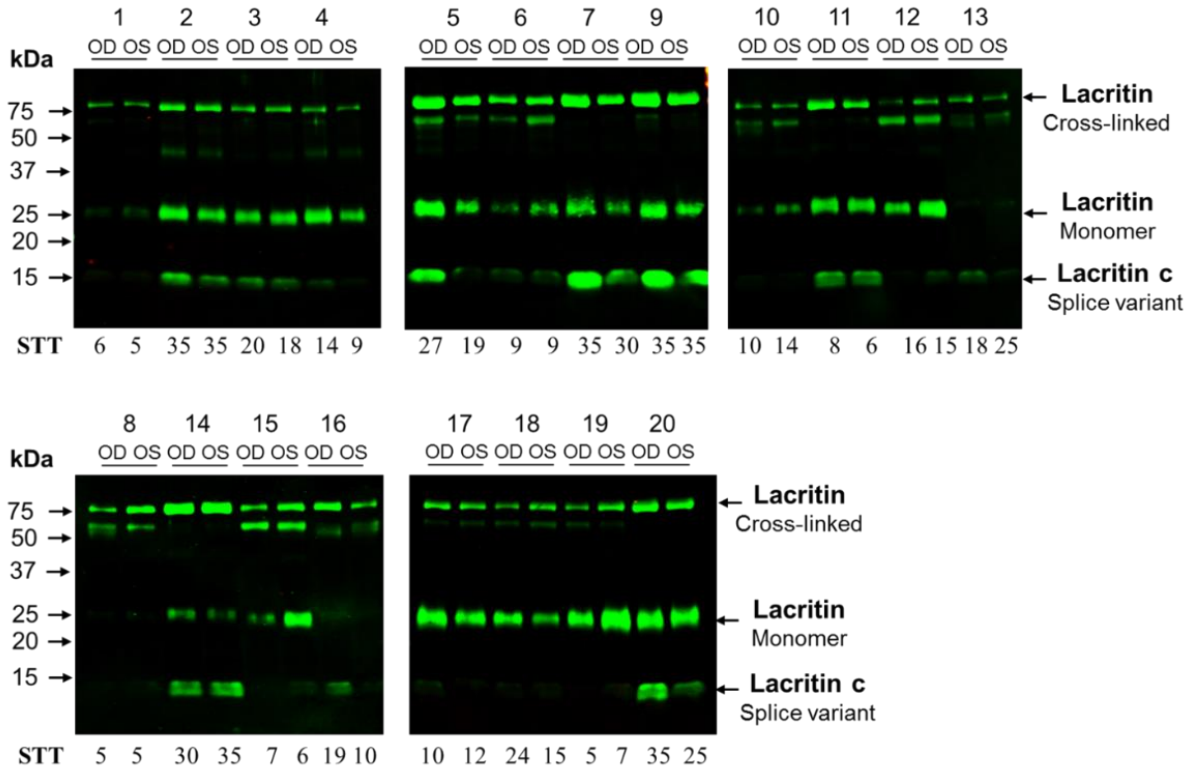
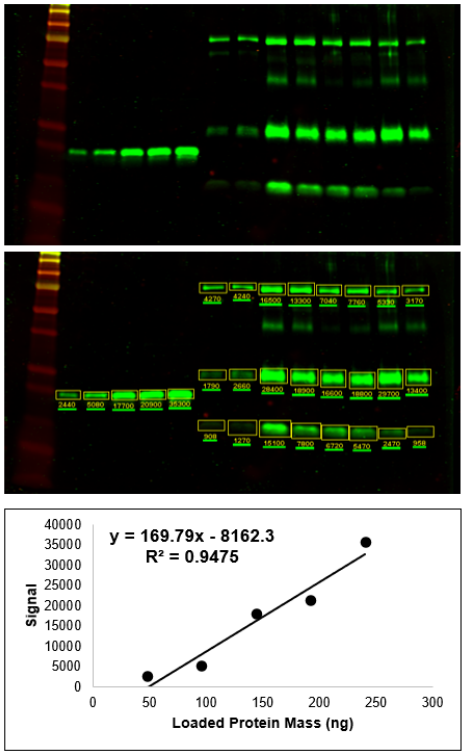


Figure 4. Western Analysis of Lacritin Isoforms from 20 Adults. Lacritin isoforms from tear samples of both right (OD) and left (OS) eyes were detected through western blots for samples 101-120. Isoforms are denoted to the right with corresponding molecular weights in kDa denoted to the left: cross-linked lacritin (75 kDa), monomeric lacritin (25 kDa), and splice variant lacritin (15 kDa). Schirmer tear test values (STT) are given for each sample (mm absorbed) in their corresponding lane at the bottom of each western blot.

Quantitation of Tear Lacritin Isoforms from 20 Adults

To quantify the nanograms and percent lacritin isoforms, western blot images obtained from the LiCor Odyssey CLx imaging system were analyzed using ImageSoftwareLite. This produced values for each western band using relative fluorescent signals obtained at the 800nm wavelength (Figure 5). Relative signals of decreasing concentrations of recombinant lacritin run on each gel were used to generate a standard curve (Figure 5). The standard curve allowed for the quantitation of total lacritin and each lacritin isoform in nanograms (Figure 5), which were then used in conjunction with total eluted protein to determine total lacritin (ng) and the percent of each

lacritin isoform present within each sample (Figure 5). Western blots of 40 tear samples and recombinant lacritin showing the relative signals is in Appendix B. An example is given of the quantitation and a summary table of the data generated for samples 101-104 (OD and OS) (Figure 5).



Normal Tear Samples 101-104
Image ID: 498_02, Blot Date 12-1-18

Sample Number	101	101	102	102	103	103	104	104
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	6	5	35	35	20	18	14	9
Eluted Protein (mg/mL)	1.27	1.02	2.59	3.07	1.8	1.86	2.46	2.14
Signal Splice Variant	908	1270	15100	7800	6720	5470	2470	958
ng Splice Variant	53	56	137	94	88	80	63	54
% Splice Variant	2.09	2.17	5.34	3.67	3.42	3.14	2.45	2.1
µM Splice Variant	33	33	40	27	26	26	37	46
Signal Monomer	1790	2660	28400	18900	16600	18800	29700	13400
ng Monomer	59	64	216	159	146	159	223	127
% Monomer	2.29	2.49	8.42	6.22	5.69	6.2	8.72	4.97
µM Monomer	32	33	54	40	37	44	114	96
Signal Cross-Linked	4270	4240	16500	13300	7040	7760	5330	3170
ng Cross-Linked	73	73	145	127	90	94	79	67
% Cross-Linked	2.86	2.85	5.68	4.95	3.5	3.66	3.1	2.61
µM Cross-Linked	7	7	6	6	4	5	7	9

Figure 5. Quantitation of Tear Samples 101 – 104. Lacritin isoforms from both the right (OD) and left (OS) eyes in each sample, as well as, recombinant lacritin standards were quantitated using relative signal to generate a standard curve. This was compared to total eluted protein to determine molarity, nanograms, and percent lacritin in each sample expressed as ng lacritin per 100ng total protein.

Distribution of Total Lacritin Isoforms from 20 Adults

To exhibit the potential for a baseline distribution of nanograms as well as percentages of lacritin isoforms, the obtained masses and percentages from the western blots were distributed in a quartile system using JMP software (Figure 6) (Figure 7). Nanograms of combined lacritin isoforms and percent lacritin of each isoform were determined from the recombinant lacritin

standard curves generated in the quantitated western blots (Appendix B). The median total lacritin in nanograms was 211.5 ng and the median total percent lacritin was 7.24% (Figure 6) (Figure 7). The 25th quartiles and 75th quartiles for total nanograms of lacritin and total percent lacritin were 136.5 ng, 326.25 ng, and 5.43%, 10.21%, respectively (Figure 6) (Figure 7). For each of the isoforms (cross-link, monomer and splice-variant) the median percentages were 2.04%, 2.435% and 1.97%, respectively (Figure 7).

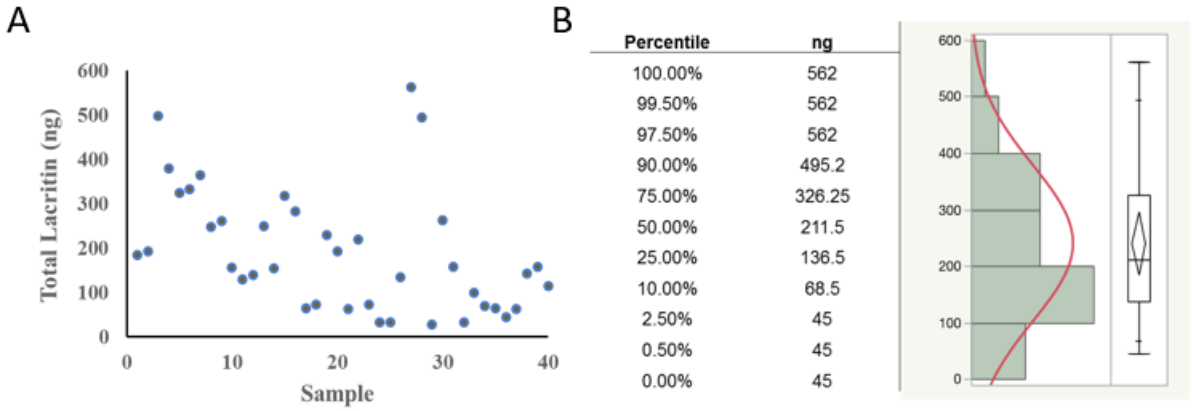


Figure 6. Distribution of Total Lacritin (ng) for Samples 101-120. The distribution of total lacritin (ng) **(A)** and the distribution of total lacritin (ng) by quartile system **(B)** are given. The standard deviation is represented to the right of the curve along with tick marks denoting the 5th and 95th percentiles of the curve. Additionally, the curves are divided into quartiles (25th, 50th, and 75th) as denoted by the box to the right of the curve. The diamond within this quartile box represents the mean of the samples; the middle of the diamond is the mean and the tips of this diamond represent the certainty of the mean.

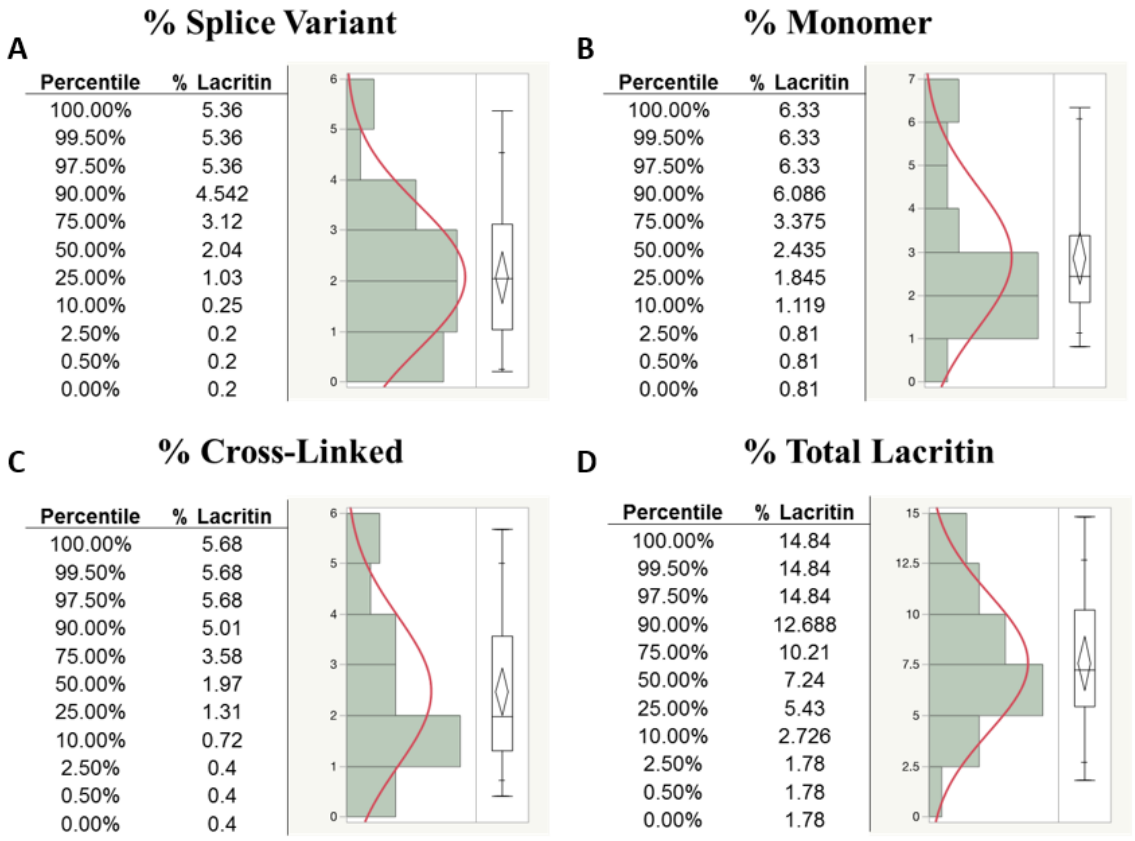


Figure 7. Distribution of Percent Lacritin Isoforms and Total Percent Lacritin for Samples 101-120. The standard deviation is represented to the right of the curves along with tick marks denoting the 5th and 95th percentiles of the curve. Additionally, the curves are divided into quartiles (25th, 50th, and 75th) as denoted by the box to the right of the curve. The diamond within this quartile box represents the mean of the samples, the middle of the diamond is the mean and the tips of this diamond represent the certainty of the mean.

DISCUSSION

A multiplex western blot immunoassay that detects three isoforms of human tear lacritin was validated for future use in human clinical trials. Quantitation of the lacritin isoforms and recombinant lacritin with the LI-COR Odyssey CLx imaging system was used to calculate nanogram and percent total protein concentrations of three lacritin isoforms found in human tears. These masses and percentages were distributed in an example baseline distribution which

could potentially provide diagnosis for lacritin-deficient dry eye. This immunoassay will be used in a Lacripep™ clinical trial to determine the distribution of tear lacritin isoforms---before and after topical application of a lacritin peptide on dry eye patients.

Validation of this diagnostic western blot assay is essential in demonstrating the potential use of lacritin as a biomarker for dry eye associated diseases. Application of this assay on tear samples obtained from patients suffering from dry eye may provide a diagnostic test for future clinical use. N-Term and I3 antibodies demonstrated that three distinct lacritin isoforms may be visualized (Figure 1). All three isoforms of lacritin were then detectable in western blots using those anti-lacritin primary antibodies followed by fluorescent antibodies (Figure 4). Additionally, all three lacritin isoforms were quantifiable using the LI-COR Odyssey CLx imaging system which was used to calculate nanograms as well as percentages of lacritin from a recombinant lacritin standard curve run on the same blot (Figure 5). This allowed for the distribution of these isoforms using a quartile system with JMP Software (Figure 7). Thus, the application of a diagnostic western blot immunoassay was validated and the potential for lacritin as a diagnostic biomarker for dry eye was demonstrated.

Tear film is a complex mixture of 183 extracellular proteins of which 4-5% of these proteins are related to of dry eye disease (Laurie et al., 2008). By placing emphasis on ocular proteins, protein therapeutics may be developed to treat the cause, rather than the symptoms of ocular diseases, like dry eye. This is particularly true when studying the roles of proteins like lacritin in the tear proteome. Lacritin has been shown to be deficient in varying forms of dry eye, most notably, Sjögren's syndrome dry eye (Karnati et al., 2013). When present in sufficient amounts, lacritin increases basal tear secretion and has cytoprotective and mitogenic properties (Ma et al., 2008). Therefore, the use of lacritin as a biomarker could make the diagnosis of

lacritin-deficient ocular disease possible and allow for the prescription of topical lacritin treatments. A novel therapeutic, Lacripep™, is currently being analyzed in a human clinical trial as an effective treatment for dry eye.

Lacripep™ utilizes a synthetic 19 amino acid derivative of lacritin as a topical therapeutic for a study in SS dry eye patients. After meeting exclusion and inclusion criteria, 201 patients, as well as age-matched controls, were recruited for a clinical trial (TearSolutions, 2017). In a triple-blind study to identify the efficacy of differing strengths of Lacripep™, 201 patients were divided into three groups, receiving either one of two Lacripep™ ophthalmic solutions of differing strengths or a placebo (TearSolutions, 2017). Patients were administered their designated ophthalmic solution over four weeks, three times daily (TearSolutions, 2017). Samples from these patients and age-matched controls were then collected on Schirmer strips and shipped to James Madison University, where analysis of the tear samples will take place. The multiplex western blot immunoassay will be used to identify the distribution of percent lacritin isoforms in a quartile system, providing a baseline for future diagnosis of dry eye patients.

Throughout this study a multiplex western blot assay was developed for the use in human clinical trials to diagnose lacritin-deficient dry eye. By properly diagnosing dry eye patients using lacritin as a protein biomarker, a lacritin therapeutic, Lacripep™, can be administered to patients to treat the cause rather than the symptoms of their specific form of dry eye. Therefore, the validated diagnostic western blot assay will contribute to the improvement of dry eye diagnosis, providing many patients with relief from a disease with no previous consensus on diagnosis and treatment.

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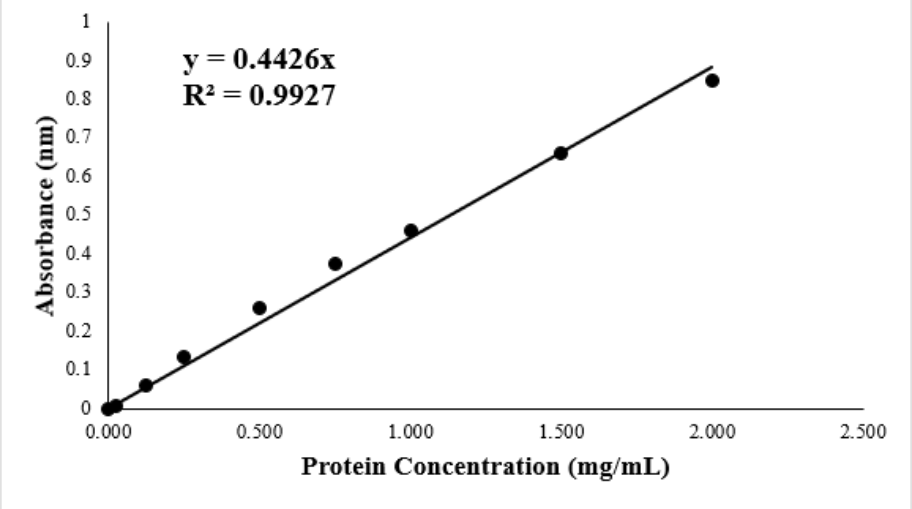
APPENDIX A

BCA 11-27-18 101-104 STDS

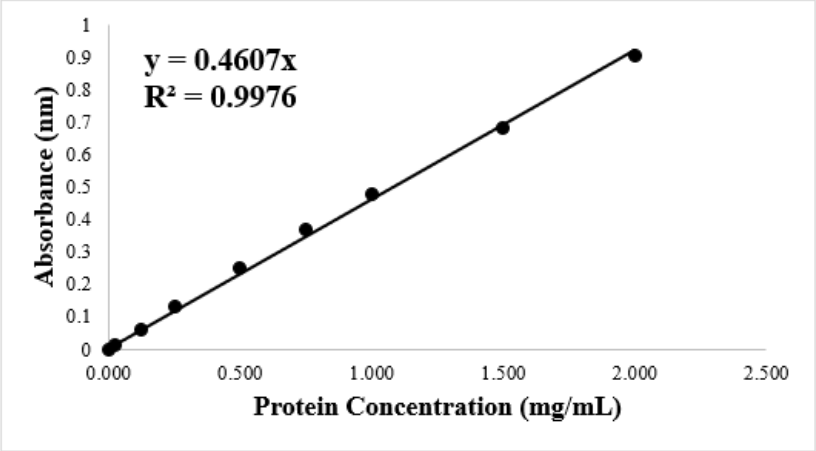
Standard	ABS 1	ABS 2	AVG	STDEV	Conc (mg/mL)	Corrected
A	0.946	0.927	0.9365	0.013435	2.000	0.85
B	0.747	0.748	0.7475	0.0007071	1.500	0.661
C	0.551	0.544	0.5475	0.0049497	1.000	0.461
D	0.455	0.463	0.459	0.0056569	0.750	0.3725
E	0.348	0.348	0.348	0	0.500	0.2615
F	0.218	0.218	0.218	0	0.250	0.1315
G	0.149	0.141	0.145	0.0056569	0.125	0.0585
H	0.095	0.095	0.095	0	0.025	0.0085
I	0.088	0.085	0.0865	0.0021213	0.000	0



Sample	ABS 1	ABS 2	AVG	STDEV	Corrected	mg/mL	ug/mL
101 OD	0.649	0.649	0.649	0	0.5625	1.27	1270.90
101 OS	0.528	0.552	0.54	0.0169706	0.4535	1.02	1024.63
102 OD	1.236	1.226	1.231	0.0070711	1.1445	2.59	2585.86
102 OS	1.395	1.498	1.4465	0.072832	1.36	3.07	3072.75
103 OD	0.894	0.875	0.8845	0.013435	0.798	1.80	1802.98
103 OS	0.88	0.941	0.9105	0.0431335	0.824	1.86	1861.73
104 OD	1.164	1.188	1.176	0.0169706	1.0895	2.46	2461.59
104 OS	0.935	1.135	1.035	0.1414214	0.9485	2.14	2143.02

BCA 11-27-18 105-107 and 109 STDS

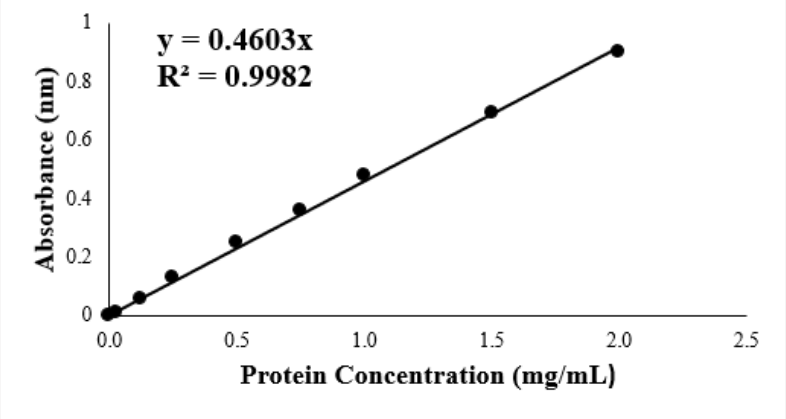
Standard	ABS 1	ABS 2	AVG	STDEV	Conc (mg/mL)	Corrected
A	0.996	0.981	0.9885	0.0106066	2.000	0.9035
B	0.754	0.781	0.7675	0.01909188	1.500	0.6825
C	0.566	0.557	0.5615	0.00636396	1.000	0.4765
D	0.46	0.449	0.4545	0.00777817	0.750	0.3695
E	0.339	0.331	0.335	0.00565685	0.500	0.25
F	0.221	0.214	0.2175	0.00494975	0.250	0.1325
G	0.148	0.144	0.146	0.00282843	0.125	0.061
H	0.095	0.097	0.096	0.00141421	0.025	0.011
I	0.085	0.085	0.085	0	0.000	0



Sample	ABS 1	ABS 2	AVG	STDEV	Corrected	mg/mL	ug/mL
105 OD	1.259	1.485	1.372	0.15980613	1.287	2.79	2793.57
105 OS	0.991	1.082	1.0365	0.06434672	0.9515	2.07	2065.34
106 OD	0.523	0.527	0.525	0.00282843	0.44	0.96	955.07
106 OS	0.706	0.698	0.702	0.00565685	0.617	1.34	1339.27
107 OD	0.93	0.945	0.9375	0.0106066	0.8525	1.85	1850.44
107 OS	0.727	0.772	0.7495	0.03181981	0.6645	1.44	1442.37
109 OD	1.158	1.148	1.153	0.00707107	1.068	2.32	2318.21
109 OS	0.929	0.932	0.9305	0.00212132	0.8455	1.84	1835.25

BCA 11-29-18 110-113

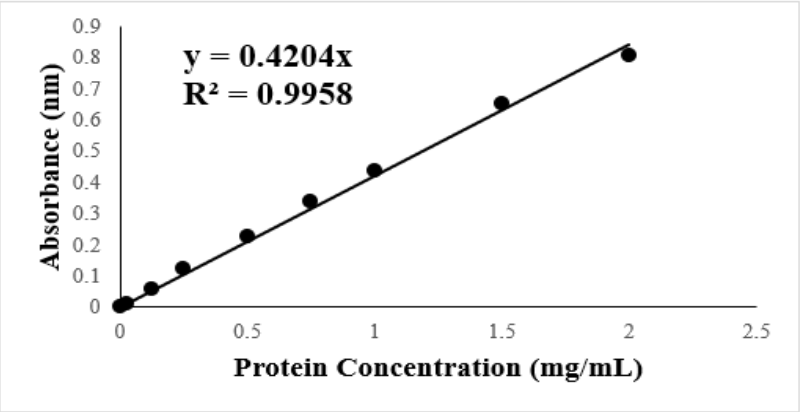
Standard	ABS 1	ABS 2	AVG	STDEV	Conc (mg/mL)	Corrected
A	1.013	0.954	0.9835	0.0417193	2.0	0.9
B	0.76	0.788	0.774	0.01979899	1.5	0.6905
C	0.568	0.554	0.561	0.00989949	1.0	0.4775
D	0.451	0.437	0.444	0.00989949	0.8	0.3605
E	0.335	0.328	0.3315	0.00494975	0.5	0.248
F	0.214	0.209	0.2115	0.00353553	0.3	0.128
G	0.142	0.141	0.1415	0.00070711	0.1	0.058
H	0.093	0.093	0.093	0	0.0	0.0095
I	0.083	0.084	0.0835	0.00070711	0.0	0



Sample	ABS 1	ABS 2	AVG	STDEV	Corrected	mg/mL	ug/mL
110 OD	0.668	0.68	0.674	0.00848528	0.5905	1.28	1282.86
110 OS	0.919	0.923	0.921	0.00282843	0.8375	1.82	1819.47
111 OD	0.665	0.649	0.657	0.01131371	0.5735	1.25	1245.93
111 OS	0.673	0.683	0.678	0.00707107	0.5945	1.29	1291.55
112 OD	0.967	0.958	0.9625	0.00636396	0.879	1.91	1909.62
112 OS	1.003	0.939	0.971	0.04525483	0.8875	1.93	1928.09
113 OD	1.01	1.007	1.0085	0.00212132	0.925	2.01	2009.56
113 OS	1.029	1.056	1.0425	0.01909188	0.959	2.08	2083.42

BCA 11-29-18 108&-114-116

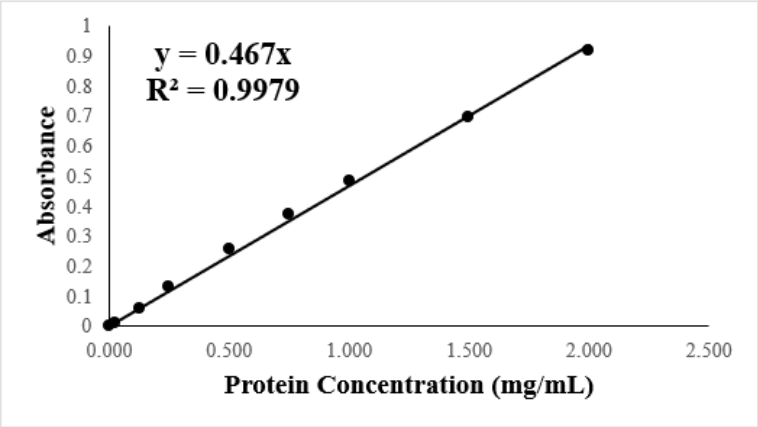
Standard	ABS 1	ABS 2	AVG	STDEV	Conc (mg/mL)	Corrected
A	0.894	0.881	0.8875	0.009192	2.000	0.805
B	0.741	0.725	0.733	0.011314	1.500	0.6505
C	0.518	0.516	0.517	0.001414	1.000	0.4345
D	0.423	0.415	0.419	0.005657	0.750	0.3365
E	0.306	0.307	0.3065	0.000707	0.500	0.224
F	0.205	0.203	0.204	0.001414	0.250	0.1215
G	0.14	0.136	0.138	0.002828	0.125	0.0555
H	0.093	0.092	0.0925	0.000707	0.025	0.01
I	0.083	0.082	0.0825	0.000707	0.000	0



Sample	ABS 1	ABS 2	AVG	STDEV	Corrected	mg/mL	ug/mL
108 OD	1.089	1.063	1.076	0.018385	0.9935	2.36	2363
108 OS	0.706	0.726	0.716	0.014142	0.6335	1.51	1507
114 OD	1.148	1.128	1.138	0.014142	1.0555	2.51	2511
114 OS	1.079	1.092	1.0855	0.009192	1.003	2.39	2386
115 OD	1.012	0.981	0.9965	0.02192	0.914	2.17	2174
115 OS	1.17	1.169	1.1695	0.000707	1.087	2.59	2586
116 OD	1.039	1.035	1.037	0.002828	0.9545	2.27	2270
116 OS	0.76	0.759	0.7595	0.000707	0.677	1.61	1610

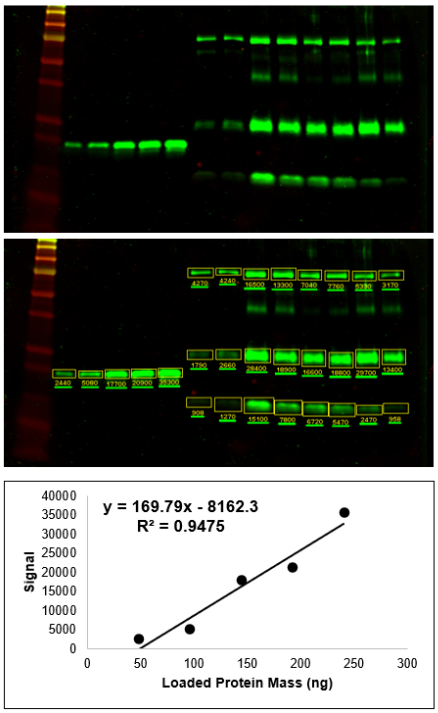
BCA 12-4-18 117-120

Standard	ABS 1	ABS 2	AVG	STDEV	Conc (mg/mL)	Corrected
A	1.01	0.989	0.9995	0.01484924	2.000	0.916
B	0.764	0.793	0.7785	0.0205061	1.500	0.695
C	0.595	0.534	0.5645	0.04313351	1.000	0.481
D	0.46	0.448	0.454	0.00848528	0.750	0.3705
E	0.35	0.331	0.3405	0.01343503	0.500	0.257
F	0.217	0.211	0.214	0.00424264	0.250	0.1305
G	0.146	0.14	0.143	0.00424264	0.125	0.0595
H	0.091	0.095	0.093	0.00282843	0.025	0.0095
I	0.086	0.081	0.0835	0.00353553	0.000	0



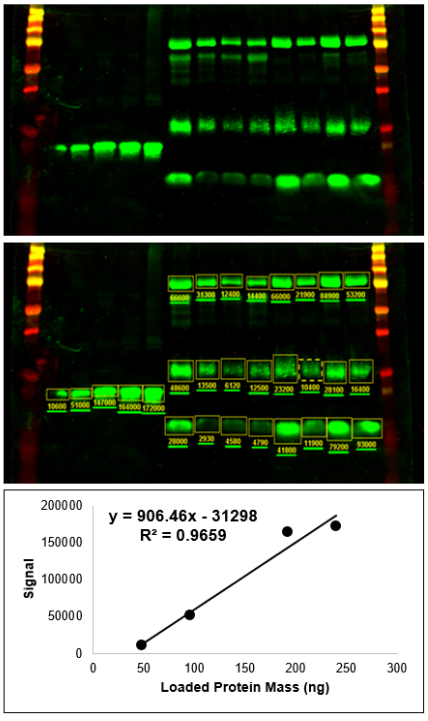
Sample	ABS 1	ABS 2	AVG	STDEV	Corrected	mg/mL	ug/mL
117OD	0.91	0.883	0.8965	0.01909188	0.813	1.74	1740.90
117OS	0.739	0.727	0.733	0.00848528	0.6495	1.39	1390.79
118OD	1.096	1.105	1.1005	0.00636396	1.017	2.18	2177.73
118OS	0.985	0.981	0.983	0.00282843	0.8995	1.93	1926.12
119OD	0.779	0.791	0.785	0.00848528	0.7015	1.50	1502.14
119OS	0.922	0.921	0.9215	0.00070711	0.838	1.79	1794.43
120OD	1.226	1.234	1.23	0.00565685	1.1465	2.46	2455.03
120OS	1.156	1.167	1.1615	0.00777817	1.078	2.31	2308.35

APPENDIX B



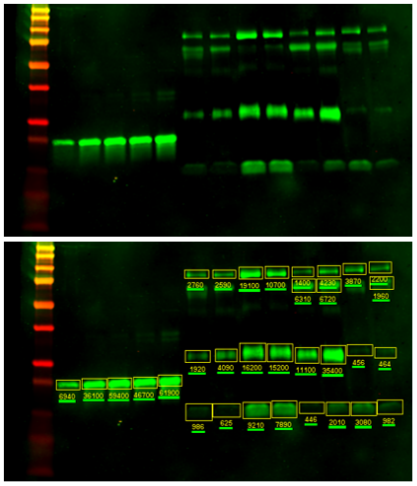
Normal Tear Samples 101-104
Image ID: 498_02, Blot Date 12-1-18

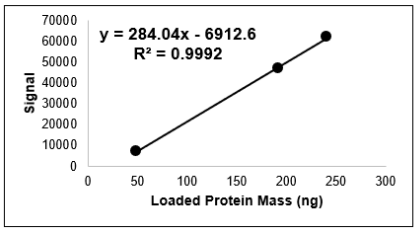
Sample Number	101	101	102	102	103	103	104	104
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	6	5	35	35	20	18	14	9
Eluted Protein (mg/mL)	1.27	1.02	2.59	3.07	1.8	1.86	2.46	2.14
Signal Splice Variant	908	1270	15100	7800	6720	5470	2470	958
ng Splice Variant	53	56	137	94	88	80	63	54
% Splice Variant	2.09	2.17	5.34	3.67	3.42	3.14	2.45	2.1
µM Splice Variant	33	33	40	27	26	26	37	46
Signal Monomer	1790	2660	28400	18900	16600	18800	29700	13400
ng Monomer	59	64	216	159	146	159	223	127
% Monomer	2.29	2.49	8.42	6.22	5.69	6.2	8.72	4.97
µM Monomer	32	33	54	40	37	44	114	96
Signal Cross-Linked	4270	4240	16500	13300	7040	7760	5330	3170
ng Cross-Linked	73	73	145	127	90	94	79	67
% Cross-Linked	2.86	2.85	5.68	4.95	3.5	3.66	3.1	2.61
µM Cross-Linked	7	7	6	6	4	5	7	9



Normal Tear samples 105-107 & 109 RS
Image ID: 523_02 Blot Date 1-4-19

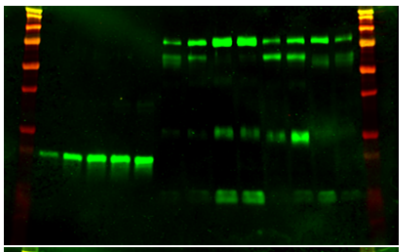
Sample Number	105	105	106	106	107	107	109	109
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	27	19	9	9	35	30	35	35
Eluted Protein (mg/mL)	2.79	2.07	0.96	1.34	1.85	1.44	2.32	1.84
Signal Splice Variant	28000	2930	4580	4790	41800	11900	79200	93000
ng Splice Variant	65	38	40	40	81	48	122	137
% Splice Variant	2.55	1.47	1.55	1.56	3.15	1.86	4.76	5.36
µM Splice Variant	23	17	14	17	16	10	35	26
Signal Monomer	48600	13500	6120	12500	23200	10400	28100	16400
ng Monomer	88	49	41	49	61	47	67	53
% Monomer	3.44	1.93	1.61	1.9	2.39	1.83	2.62	2.08
µM Monomer	27	20	12	18	10	9	17	9
Signal Cross-Linked	66600	31300	12400	14400	66000	21900	84900	53200
ng Cross-Linked	108	69	48	50	107	59	128	93
% Cross-Linked	4.22	2.7	1.88	1.97	4.19	2.29	5.01	3.64
µM Cross-Linked	6	5	3	3	3	2	6	3

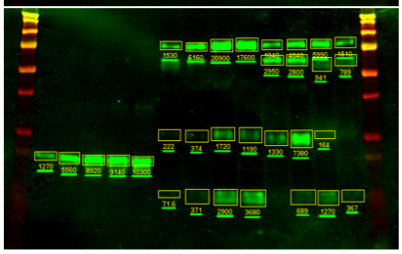


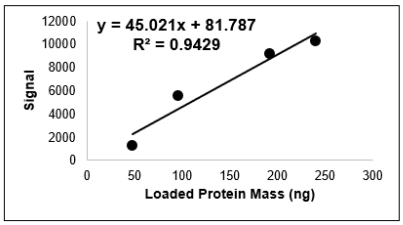


Normal Tear Samples 110-113
Image ID: 512_02 Blot Date 12-13-18

Sample Number	110	110	111	111	112	112	113	113
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	10	14	8	6	16	15	18	25
Eluted Protein (mg/mL)	1.28	1.82	1.25	1.29	1.91	1.93	2.01	2.08
Signal Splice Variant	986	625	9210	7890	446	2010	3080	982
ng Splice Variant			57	52		31	35	
% Splice Variant			2.22	2.04		1.23	1.37	
µM Splice Variant			27	33		17	17	
Signal Monomer	1920	4090	16200	15200	11100	35400	456	464
ng Monomer	31	39	81	78	63	149		
% Monomer	1.21	1.51	3.18	3.04	2.48	5.82		
µM Monomer	11	13	34	42	19	72		
Signal Cross-Linked	2760	2590	19100	10700	1400	4230	3870	2200
ng Cross-Linked	34	33	92	62		40	38	32
% Cross-Linked	1.33	1.31	3.58	2.42		1.53	1.48	1.25
µM Cross-Linked	2	2	7	6		3	3	2

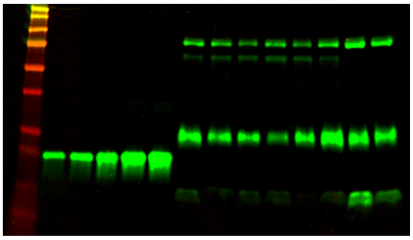


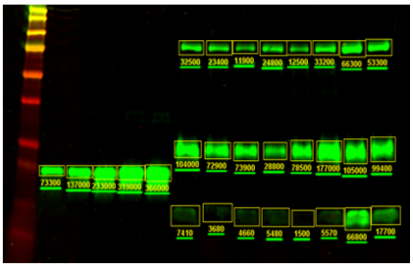


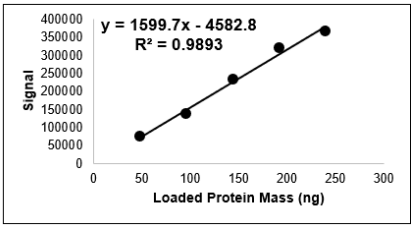


Normal Tear Samples 108 & 114-116
ID: 510_02, Blot Date 12-13-18

Sample Number	108	108	114	114	115	115	116	116
Data	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	5	5	30	35	7	6	19	10
Total Protein (mg)	2.36	1.51	2.51	2.39	2.17	2.59	2.27	1.61
Signal Splice Variant			2900	3680		689	1270	367
ng Splice Variant			63	80		13	26	
% Splice Variant			2.44	3.12		0.53	1.03	
µM Splice Variant			20	23		17	12	
Signal Monomer			1720	1190	1330	7380		
ng Monomer			36	25	28	162		
% Monomer			1.42	0.96	1.08	6.33		
µM Monomer			10	6	26	176		
Signal Cross-Linked	1530	6160	20900	17600	1840	4040	5990	1510
ng Cross-Linked	32	135	463	389	39	88	131	32
% Cross-Linked	1.25	5.27	18.10	15.20	1.53	3.44	5.12	1.24
µM Cross-Linked	7	20	23	17	7	17	9	3







Normal Tear Samples 117-120
ID: 513_01, Blot Date 12-13-18

Sample Number	117	117	118	118	119	119	120	120
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
STT (mm)	12	10	24	15	5	7	35	25
Eluted Protein (mg/mL)	1.74	1.39	2.18	1.93	1.5	1.79	2.46	2.31
Signal Splice Variant	7410	3680	4660	5480		5570	66800	17700
ng Splice Variant	8	5	6	6		6	45	14
% Splice Variant	0.29	0.2	0.23	0.25		0.25	1.74	0.54
µM Splice Variant	3	2	2	2		5	13	5
Signal Monomer	10400	72900	73900	28800	78500	177000	105000	99400
ng Monomer	68	48	49	21	52	113	69	65
% Monomer	2.64	1.89	1.92	0.81	2.03	4.43	2.68	2.54
µM Monomer	26	17	16	7	44	71	17	21
Signal Cross-Linked	32500	23400	11900	24800	12500	33200	66300	53300
ng Cross-Linked	23	17	10	18	11	24	44	36
% Cross-Linked	0.91	0.68	0.4	0.72	0.42	0.92	1.73	1.41
µM Cross-Linked	2	1	1	1	2	3	2	2