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Development of a Quantitative Immunoassay for Tear Lacritin Proteoforms

Brooke M. Justis[1], Casey E. Coburn[1], Ethan M. Tyler[1], Ryan S. Showalter[1], Brianna J. Dissler[1], Melissa Li[2], Nancy A. McNamara[2,3], Gordon W. Laurie[4–6], and Robert L. McKown[1]

[1] School of Integrated Sciences, James Madison University, Harrisonburg, VA USA
[2] School of Optometry and Vision Science Program, University of California, Berkeley, Berkeley, CA, USA
[3] Department of Anatomy, University of California, San Francisco, San Francisco, CA, USA
[4] Department of Cell Biology, University of Virginia, Charlottesville, VA, USA
[5] Department of Ophthalmology, University of Virginia, Charlottesville, VA, USA
[6] Department of Biomedical Engineering, University of Virginia, Charlottesville, VA, USA

Correspondence: Robert L. McKown, School of Integrated Sciences, James Madison University, 701 Carrier Drive, Harrisonburg, VA 22807, USA. e-mail: mckownrl@jmu.edu

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Purpose: Lacritin is a tear glycoprotein with pro-tearing and pro-ocular surface homeostasis activities that is selectively deficient in most dry eye tears. Proteoforms include an active monomer, inactive polymers, and a splice variant termed lacritin-c. Quantitation of the different proteoforms of tear lacritin may provide a diagnostic tool for ocular diseases. Here, we report the development of an immunoassay for the quantification of multiple lacritin proteoforms in human tear samples.

Methods: Basal tears collected on Schirmer test strips with anesthesia were eluted by diffusion and centrifugation under optimized conditions. Tear protein concentrations were determined, and 2.56 µg of each sample was separated by SDS-PAGE followed by western blot analysis. Blots were challenged with anti-Pep Lac N-term antibodies. Detection was with fluorescent secondary antibodies visualized by the LI-COR Odyssey CLx imaging system and quantified with standard curves of recombinant lacritin.

Results: The percent total lacritin (ng lacritin/100 ng total protein) ranged from 1.8% to 14.8%. Monomer, lacritin-c, and polymer proteoform percent total protein ranged from 1.1% to 6.3%, 0.3% to 5.4%, and 0.7% to 5.7%, respectively. Monomer lacritin was detected at concentrations of 6 to 176 µM, with lacritin-c and polymer proteoforms at 2 to 46 µM and 1 to 23 µM, respectively.

Conclusions: This assay greatly exceeds the power and sensitivity of our prior lacritin enzyme-linked immunosorbent assay that was not capable of distinguishing monomer from polymers and lacritin-c proteoforms.

Translational Relevance: A new method has been developed to quantitate multiple proteoforms of tear lacritin in preparation for analyses of samples from clinical trials.

Introduction

A healthy ocular surface is maintained in large part by a complex mixture of biologically active proteins found in tear film. A thick aqueous layer composed of water, electrolytes, and a diverse collection of proteins, peptides, and glycoproteins is primarily secreted from the lacrimal glands. The tear film lubricates the ocular surface, nourishes avascular tissues, and provides a protective antimicrobial barrier. It has been estimated that the tear proteome is composed of approximately 1500 proteins.[1] Although most of these proteins are considered intracellular as a consequence of cell death from normal epithelial renewal, over 200 proteins have been predicted to be extracellular with biological functions that include proangiogenic, anti-angiogenic, retinal survival, epithelial

repair, immunosuppressive, and immunostimulatory activities.[2] Although the ocular surface is exposed to numerous pathogenic microorganisms, epithelium of the eye is rarely subject to bacterial infection.[3] One of the first tear proteins characterized was a bacteriolytic element found in tears termed lysozyme.[4] Since then, a variety of antimicrobial factors have been identified in human tear film, including IgA antibodies, defensins, histatins, cathelicidin LL-37, lipocalin 2, and a cleavage-potentiated fragment of tear lacritin.[5] The concentrations and relative distribution of tear proteins define a metabolic state of the ocular surface, and a stable balance of these proteins maintains a healthy homeostasis for the outer eye. Disruption of the delicate balance of tear proteins from genetic, metabolic, and environmental factors has been associated with the onset of ocular diseases. Identification and biochemical analysis of tear proteins have provided a tool for the diagnosis and potential treatment of ocular diseases. A number of noninvasive methods for the collection and analysis of tear samples in a relatively cell-free environment have been evaluated that utilize ophthalmic sponges, capillary tubes, and Schirmer test strips.[6] Reviews of the tear proteome with references to methods of collection, biochemical analysis techniques, and correlations to ocular diseases have been published.[7]

Comparative tear protein profile analysis of tears collected on ophthalmic sponges from dry eye and control individuals indicated changes in the expression levels in a number of proteins.[8] Although tear collection with ophthalmic sponges has the advantage that they are well tolerated by the patient because tears are collected from the lower eyelid, biochemical analysis can yield diverse results with different sponges, extraction protocols, and specific proteins targeted for analysis.[9] In a comparison of total tear protein content recovered from Schirmer test strips and ophthalmic sponges, Schirmer test strips were shown to be more reliable in producing statistically significant data.[10] Tears can be collected directly via capillary action with glass capillary tubes carefully placed at the lower tear meniscus. Tears collected using capillary tubes and Schirmer test strips were analyzed for total protein and selected tear proteins by western blot analysis. Comparison of these collection techniques showed that Schirmer test strips have several advantages over the capillary tubes for collection and analysis of tear proteins.[11] Schirmer test strips are sterile strips of filter paper placed inside the lower eyelid for 5 minutes and the amount of tears adsorbed can be recorded by observing the leading edge of moisture printed in millimeters on the strip.

The Schirmer tear test (STT) is a routinely used diagnostic test for dry eye and tears eluted from the strips have been used for biochemical analysis. Tears collected on Schirmer test strips cut into pieces, digested with trypsin, and analyzed using a liquid chromatography–Quadrupole-Orbitrap mass spectrometry system (Thermo Fisher Scientific, Waltham, MA) revealed that 18 proteins were differentially expressed in dry eye patients.[12] Over 1000 proteins were identified using one-dimensional liquid chromatography–tandem mass spectrometry (LC-MS/MS) combined with two-dimensional LC-MS/MS from tears collected on Schirmer test strips.[13] Two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry was used to analyze tears collected on Schirmer test strips from postmenopausal women with dry eye disease.[14] Proteomic analysis of tear proteins eluted from Schirmer test strips that combine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with various spectrometric technologies has been reviewed.[15] The development of a microfluidic homogeneous immunoassay capable of making rapid, quantitative, and specific measurements of endogenous tear protein biomarkers in human tear fluid eluted from Schirmer test strips has been reported.[16] Tear collection on Schirmer test strips combined with a diffusion-based extraction is an established methodology for protein analysis.[17–20] It should be noted that there is no standardization of commercial Schirmer test strips, and considerable variations on tear collection and analysis have been observed among various commercial brands. In a recent study, significant differences among five commercial brands of Schirmer test strips were found in uptake, release volume, and protein retention.[21] Therefore, if Schirmer test strips are utilized for elution and analysis of tear proteins, control experiments should be done to determine the physical parameters of the Schirmer test strips employed.

Lacritin is an endogenous tear glycoprotein released from human lacrimal acinar cells that was discovered in a cDNA screen of expressed lacrimal proteins and identified as a novel secretion-enhancing factor.[22] Mature monomeric lacritin is composed of 119 amino acids with a molecular weight of 12.3 kDa; it contains a cationic amphipathic alpha-helix in the C-terminus shown to be an active site for mitogenic and anti-inflammatory properties.[23] Lacritin can restore corneal health caused by inflammatory cytokines by altering autophagy.[24] Induced cell death of corneal epithelial cells by lipopolysaccharide was alleviated with the addition of lacritin.[25] Proliferation of corneal epithelial cells is mediated by the binding of lacritin to

syndecan-1 cell surface receptors generating mitogenic signaling.[26,27] Topical application of lacritin to the eyes of rabbits promotes sustained basal tearing.[28] Lacritin has been reported to be decreased in patients with various forms of dry eye disease, including dry eye disease associated with Sjögren's syndrome.[29] It has been suggested that lacritin may be a natural replacement therapy for aqueous-deficient dry eye.[2,30] Lacritin-specific antibodies have been produced, and human tear lacritin has been detected by western blot analysis as an active lacritin monomer and as inactive tissue transglutaminase cross-linked polymers that have been shown to be a negative regulator of monomeric lacritin bioactivity.[31] As reported here, a splice variant of lacritin with unique intron coding sequences and unknown functions, termed lacritin-c, has also been detected in human tear samples. An enzyme-linked immunosorbent assay protocol has been developed to evaluate lacritin levels in tear samples from healthy adults[32]; however, this assay does not distinguish among the three proteoforms of lacritin detected in human tears. Here, we report the development of a quantitative immunoassay for tear lacritin proteoforms validated with tears from 20 healthy adults in preparation for analysis of tear samples from the phase II clinical trial of Lacripep (TearSolutions, Inc., Charlottesville, VA) in subjects with dry eye associated with primary Sjögren's syndrome.

Materials and Methods

Human Subject Recruitment and Tear Sample Collection

All aspects of the human subject studies presented in this manuscript followed the tenets of the Declaration of Helsinki and were approved by the University of California, Berkeley, Committee for Human Research prior to subject recruitment. Informed consent was obtained from all subjects, and all study activities were Health Insurance Portability and Accountability Act compliant. Tear samples were collected from both eyes of 20 healthy subjects using standard Schirmer Tear Flow Strips (Gulden Ophthalmics, Elkins Park, PA). Approximately 5 minutes following the topical application of 1 drop of proparacaine hydrochloride ophthalmic solution (0.5%), Schirmer test strips were carefully inserted into the lower, temporal, palpebral cul-de-sac of both eyes for 5 minutes. After 5 minutes, the anesthetized Schirmer value was recorded in millimeters and strips were stored in individually labeled tubes at –80°C until processing for tear protein.

Recovery and Processing of Tear Proteins

Tear samples were shipped frozen on Gulden Ophthalmics Schirmer Tear Flow Strips and stored at –80°C. Schirmer test strips were thawed, placed into spin baskets (Corning Costar Spin-X Centrifuge Tube Filters without membrane; available from Thermo Fisher Scientific, Waltham, MA), inserted into microcentrifuge collection tubes; 60 µL of filter-sterilized phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) was then loaded onto the strips. Following incubation at room temperature for 20 minutes, tear samples were eluted by centrifugation at 17.0*g* for 10 minutes at 4°C. Total protein concentrations were determined with a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific), and the eluted tear samples were stored frozen at –80°C.

Correlation of Schirmer Tear Test Values and Tear Volume Collected

Increasing volumes from 2.5 µL to 25 µL of bovine serum albumin (BSA, 10 mg/mL; Sigma-Aldrich), Contrived Tears, and Contrived Tears with Lipids (Ursa BioScience, Bel Air, MD) were pipetted into individual wells of a microtiter plate and adsorbed onto Gulden Ophthalmics Schirmer test strips for 5 minutes. The resulting Schirmer tear test value (mm) was then recorded. The millimeters adsorbed (STT) were plotted against microliters of BSA or Contrived Tears adsorbed.

Recovery of Proteins from Schirmer Test Strips

Increasing volumes from 2.5 µL to 25 µL of BSA (10 µg/mL), Contrived Tears, or Contrived Tears with Lipids were adsorbed to Schirmer test strips and eluted as described in the earlier Recovery and Processing of Tear Proteins section. Contrived Tears (artificial tears), developed by Ursa BioScience for research applications, are formulated with components known to be present in tears and were used to determine any effects of salt, pH, and lipids found in normal tears on the recovery of proteins from Schirmer test strips. Protein concentrations of the eluted samples were determined by the BCA assay. Protein concentrations of samples not adsorbed were determined by the addition of 60 µL PBS to increasing volumes from 2.5 µL to 25 µL of BSA (10 µg/mL), Contrived Tears, or Contrived Tears with Lipids followed by the BCA assay. Protein concentrations (mg/mL) for proteins adsorbed/desorbed (eluted) and proteins not adsorbed/desorbed (100%) were plotted against volume adsorbed (µL). To assess

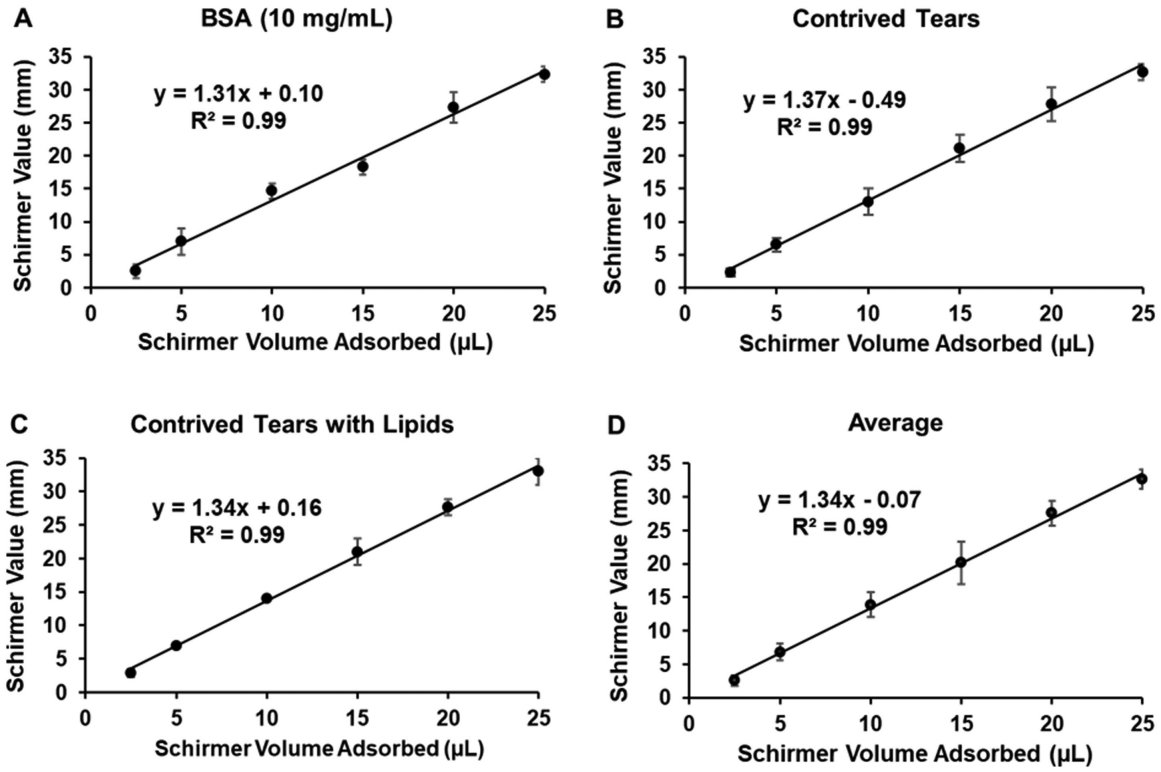


Figure 1. Schirmer tear test values and tear volume adsorbed. Shown are graphs of volumes of (A) BSA (10 mg/mL), (B), Contrived Tears, and (C) Contrived Tears with Lipids adsorbed to Schirmer test strips versus Schirmer values (mm). Each data point represents the average of three individual experiments. (D) Graph shows the average of the three combined graphs.

recovery of lacritin from Schirmer test strips, recombinant lacritin expressed and purified from bacterial cells[27] was added to BSA (10 mg/mL) and adsorbed to Schirmer test strips for 5 minutes in increasing volumes from 2.5 µL to 25 µL. Total protein was adsorbed/desorbed (eluted), and recombinant lacritin was analyzed by western blot as described below. BSA spiked with the same volumes of lacritin but not adsorbed/desorbed (100%) was analyzed on the same western blot. Recombinant lacritin of known concentrations was run on the same blot to calculate nanogram amounts of lacritin recovered.

Antibodies and Western Blotting

Anti-Pep Lac N-term antibodies were produced from the peptide EDASSDSTGADPAQEAGTS corresponding to the N-terminus of mature human lacritin amino acids 1 to 19 without signal peptide.[32] Anti-lacritin-c I3 antibodies were produced from the peptide SKSLSLCQINNLEKSLAAGPHHTSTHRDKPG[33] corresponding to 31 of the 39 amino acids contained within intron 3 of the human splice variant of lacritin termed lacritin-c. Peptides were synthesized to >85% purity and conjugated to keyhole limpet hemocyanin by Bio-Synthesis, Inc. (Lewisville, TX). New Zealand white rabbits were immunized in three boosts with anti-lacritin peptides, and the final antiserum was collected on day 70. Tear samples were thawed and diluted with PBS to 400 µg/mL, and 2.56 µg of each sample was loaded on Any kD Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad, Hercules, CA), electrophoresed at 200 V, and transferred to nitrocellulose (Protran BA83; Whatman, Dassel, Germany) in 10% ethanol, 24-mM Tris, and 19-mM glycine. Blots were blocked with PBS and 0.3% Tween 20 (PBST; Acros Organics, Fair Lawn, NJ), incubated with anti-lacritin primary antibodies (1:500 dilution in PBST) for 1 hour at room temperature, washed with PBST, and incubated for 1 hour at room temperature with goat anti-rabbit IRDye 800CW fluorescent secondary antibodies (LI-COR, Inc., Lincoln, NE). Blots were washed with PBST, air dried, wrapped in aluminum foil, and stored at 4°C.

Imaging, Quantitation, and Statistical Analysis

The LI-COR Odyssey CLx imaging system produces a signal number for each band identified on a western blot generated by the near-infrared

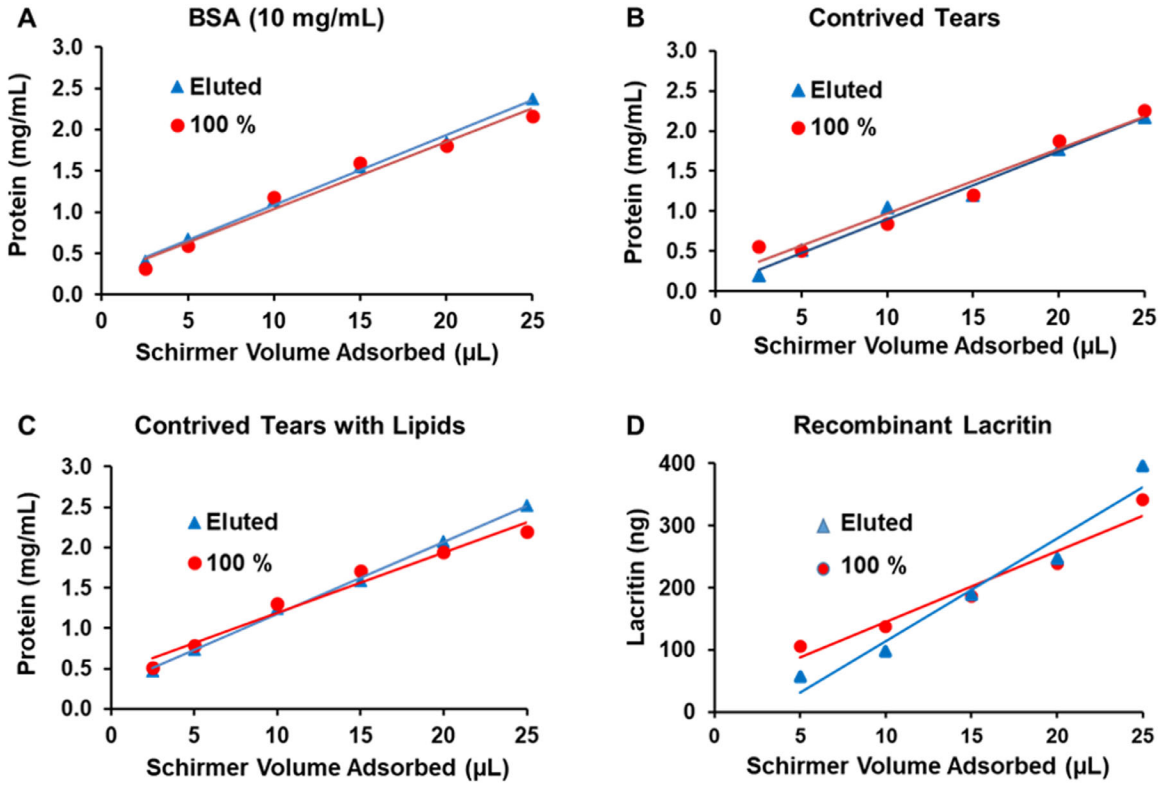


Figure 2. Recovery of proteins from Schirmer test strips. (A) BSA (10 mg/mL), (B) Contrived Tears, and (C) Contrived Tears with Lipids adsorbed to Schirmer test strips were eluted with 60 µL PBS by centrifugation. Protein concentrations were determined by the BCA assay directly with no adsorption/desorption (100%) or after adsorption/desorption from Schirmer test strips (eluted) and plotted versus volume adsorbed to the Schirmer test strips. Each data point represents the average of three individual experiments. (D) Recombinant lacritin was adsorbed to Schirmer test strips in increasing volumes from 2.5 µL to 25 µL. Total protein was eluted, and the same volumes of recombinant lacritin but not adsorbed/desorbed (100%) was analyzed on the same blot. Recombinant lacritin of known concentrations was run on the same blot to calculate the nanogram amounts of lacritin eluted versus lacritin not adsorbed.

fluorescent detection of secondary antibodies used. Tear lacritin proteins can be quantified on western blots by the generation of a standard curve from known concentrations of purified recombinant lacritin run on the same blot. Specific protein bands are quantified by near-infrared fluorescent signals and a linear regression equation generated from recombinant lacritin to approximate concentrations of tear lacritin proteins. Box plot quartile analysis was generated by JMP statistical software (SAS Institute, Cary, NC)

Results

Correlation of Schirmer Tear Test Values and Tear Volume Collected

The Schirmer tear test measurement of millimeters tears adsorbed was correlated with the volume of tears adsorbed (in µL). As shown in Figure 1, increasing volumes from 2.5 µL to 25 µL of BSA (10 mg/mL), Contrived Tears, and Contrived Tears with Lipids were adsorbed onto Schirmer test strips for 5 minutes, and the resulting Schirmer value (in mm) was recorded. All three solutions produced a similar linear relationship when plotting millimeters adsorbed as determined by the Schirmer tear test versus microliters adsorbed with a statistically significant R^2 value of 0.99. An average of the three experimental values produced a graph with a linear equation of $y = 1.34x - 0.07$ ($R^2 = 0.99$). Therefore, the volume of tears collected from Schirmer test strips can be approximated by the following equation: µL tears = mm (Schirmer value) + 0.07/1.34. Approximation of tear volumes determined by this equation can be used with nanogram lacritin measurements to determine micromolar concentrations of individual lacritin proteoforms in tear samples.

Recovery of Proteins from Schirmer Test Strips

To access recovery of proteins, increasing volumes from 2.5 µL to 25 µL of BSA (10 µg/mL), Contrived

Tears, or Contrived Tears with Lipids were adsorbed to Schirmer test strips and eluted as described in the Materials and Methods section. Protein concentrations of the eluted samples were determined by the BCA assay. Protein concentrations of samples not adsorbed were determined by the addition of 60 µL PBS to increasing volumes from 2.5 µL to 25 µL of BSA (10 µg/mL), Contrived Tears, or Contrived Tears with Lipids followed by the BCA assay. Figures 2A–2C shows graphs of protein concentrations versus volume adsorbed for proteins adsorbed/desorbed (eluted) and proteins not adsorbed/desorbed (100%). As shown in Figure 2, all samples produced a linear relationship between protein concentrations and volume adsorbed for eluted samples. Protein samples not adsorbed showed a parallel relationship when superimposed on the eluted samples; therefore, the recovery of proteins from the Schirmer test strips was approximately equal to or greater than 90% for all samples analyzed. To assess recovery of lacritin from Schirmer test strips, recombinant lacritin was added to BSA (10 mg/mL) and adsorbed to Schirmer test strips in increasing volumes from 2.5 µL to 25 µL. Total protein was eluted and recombinant lacritin analyzed by western blot as described in the Materials and Methods section. BSA spiked with the same volumes of recombinant lacritin but not adsorbed/desorbed (eluted) was analyzed on the same western blot (100%). Recombinant lacritin of known concentrations was run on the same blot to calculate nanogram amounts of lacritin recovered. Figure 2D is a graph of increasing volumes of recombinant lacritin adsorbed/desorbed (eluted) versus lacritin recovered compared to the same volumes of lacritin not adsorbed/desorbed (100%). Figure 2D is a spiking experiment designed to address the question of whether or not lacritin remains preferentially bound to Schirmer test strips following the elution protocol. Although the slight difference of more lacritin being eluted compared to lacritin not adsorbed/desorbed (100%) is an interesting observation, the experiment showed that lacritin is not preferentially bound to Schirmer test strips following the elution protocol.

Antibodies Detect Different Proteoforms of Lacritin in Human Tears

As illustrated in Figure 3A, genomic lacritin contains five coding exons separated by four introns. Exon 1 codes for the signal peptide that is cleaved off upon secretion to produce a mature monomeric protein of 119 amino acids found in human tears. A splice variant of lacritin termed lacritin-c contains

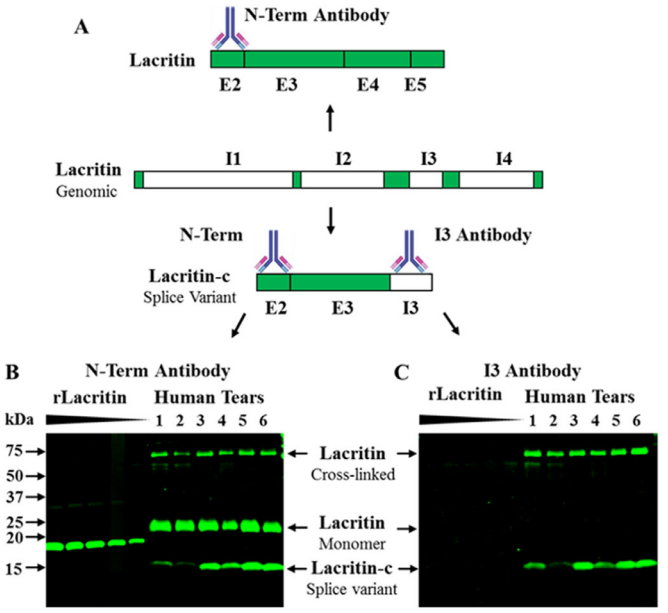


Figure 3. Antibodies detect different proteoforms of lacritin in human tears. (A) Anti-lacritin antibodies were produced against N-term sequences and unique sequences of the splice variant lacritin-c. (B) N-term antibodies detect monomeric lacritin, cross-linked polymers of lacritin, lacritin-c in human tears, and recombinant lacritin produced in bacteria by western blot analysis. (C) Antibodies unique to I3 of lacritin detect only lacritin-c and polymers of lacritin-c by western blot analysis.

exons 2 and 3 fused in frame to 39 amino acids of intron 3 to produce a splice variant protein of 104 amino acids with an unknown function. Rabbit polyclonal antibodies were produced against the first 19 amino acids of exon 2 (anti-Pep Lac N-term antibody) and 31 amino acids of intron 3 (I3 antibody). Tears collected on Schirmer test strips from healthy adults were eluted, and the total protein content was determined. Tear proteins normalized to 400 µg/mL and purified recombinant lacritin of known concentrations were separated by SDS-PAGE, transferred to nitrocellulose, and challenged by anti-lacritin antibodies. One western blot of six tear samples and recombinant lacritin was challenged with anti-lacritin N-term antibody (Fig. 3B), and a second blot with the same samples was challenged with anti-lacritin I3 antibody (Fig. 3C). Western blots were developed by fluorescent secondary antibodies and imaged by a LI-COR Odyssey CLx imaging system. Anti-lacritin N-term antibodies detected full length tear lacritin (monomer), recombinant lacritin, and lacritin-c (Fig. 1B), whereas I3 antibodies detected only lacritin-c-specific sequences (Fig. 1C). Both antibodies detected cross-linked polymer forms of lacritin (Figs. 1B, 1C).

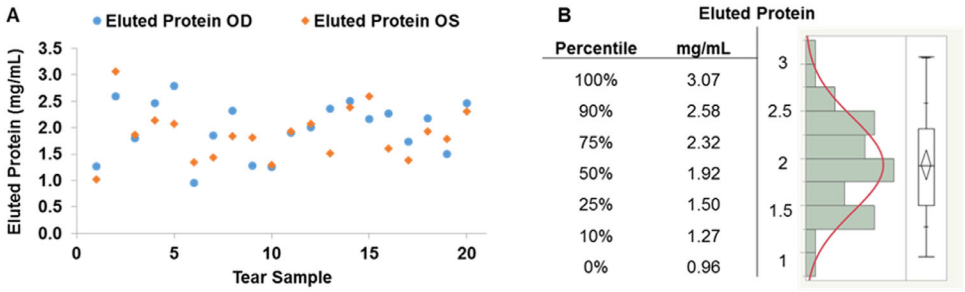


Figure 4. Eluted total protein. (A) Total protein concentrations of eluted tear samples from 20 individuals (OD and OS) as a scatterplot. (B) Box plot quartile statistical analysis of total eluted protein concentrations from 40 samples.

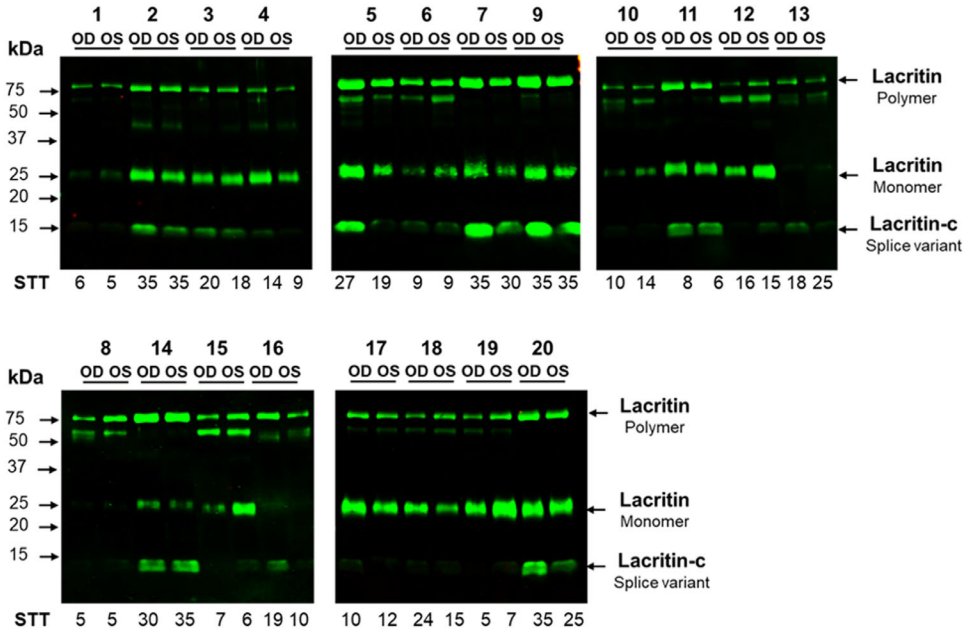


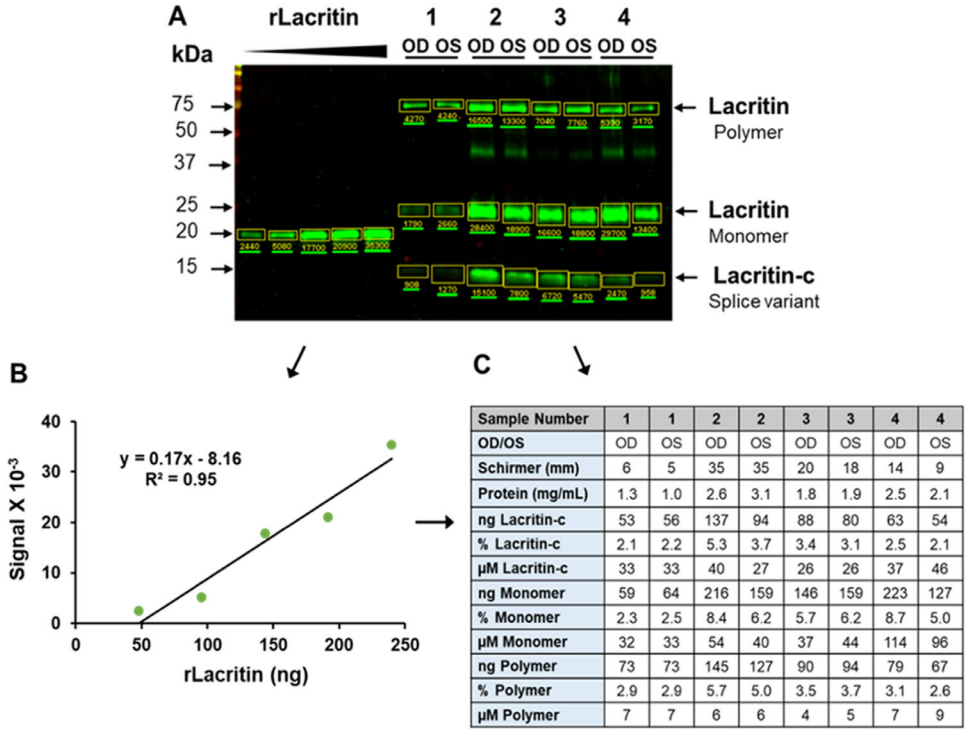
Figure 5. Western blot analysis of human tear samples challenged with anti-lacritin N-term primary antibodies and fluorescent secondary antibodies from both eyes (OD and OS) of 20 individuals. The three proteoforms of lacritin are denoted as cross-linked polymer, monomer, and the splice variant lacritin-c.

Analysis of 40 Human Tear Samples

Tear samples were collected on Schirmer test strips from both eyes of 20 healthy adults ($n = 40$), placed in cryotubes, and stored at –80°C at the site of collection. Samples were shipped to James Madison University on dry ice by overnight delivery and stored at –80°C until processed for analysis. The total protein concentration eluted from each strip was determined by the BCA assay. Figure 4A shows the distribution of total eluted protein concentrations (mg/mL) from the 20 individuals for both eyes (oculus dexter, OD; oculus sinister, OS). Figure 4B shows a box plot quartile analysis of the eluted proteins.

Western Blots and Quantitation of Lacritin Proteoforms

Tear samples collected from both eyes of 20 individuals were eluted from Schirmer test strips, separated by SDS-PAGE, transferred to nitrocellulose, and challenged with anti-lacritin N-term antibodies. Figure 5 shows a composite from five western blots of the 40 tear samples collected from both eyes (OD and OS) of 20 individuals. Also shown are the Schirmer tear test measurements (mm) of the tear samples collected. Figure 6A is an example of the numerical fluorescent signals generated for each lacritin proteoform from individuals 1 through 4. Figure 6B is the standard curve of recombinant lacritin run on



Sample Number	1	1	2	2	3	3	4	4
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
Schirmer (mm)	6	5	35	35	20	18	14	9
Protein (mg/mL)	1.3	1.0	2.6	3.1	1.8	1.9	2.5	2.1
ng Lacritin-c	53	56	137	94	88	80	63	54
% Lacritin-c	2.1	2.2	5.3	3.7	3.4	3.1	2.5	2.1
µM Lacritin-c	33	33	40	27	26	26	37	46
ng Monomer	59	64	216	159	146	159	223	127
% Monomer	2.3	2.5	8.4	6.2	5.7	6.2	8.7	5.0
µM Monomer	32	33	54	40	37	44	114	96
ng Polymer	73	73	145	127	90	94	79	67
% Polymer	2.9	2.9	5.7	5.0	3.5	3.7	3.1	2.6
µM Polymer	7	7	6	6	4	5	7	9

Figure 6. Quantitation of lacritin proteoforms from four individuals. (A) Fluorescent signals generated for each lacritin proteoform from individuals 1 through 4. (B) A standard curve of signal versus known concentrations of recombinant lacritin. (C) Summary table of data generated from eight tear samples obtained from both eyes (OD and OS) of four individuals (percent = ng lacritin proteoform/100 ng total protein).

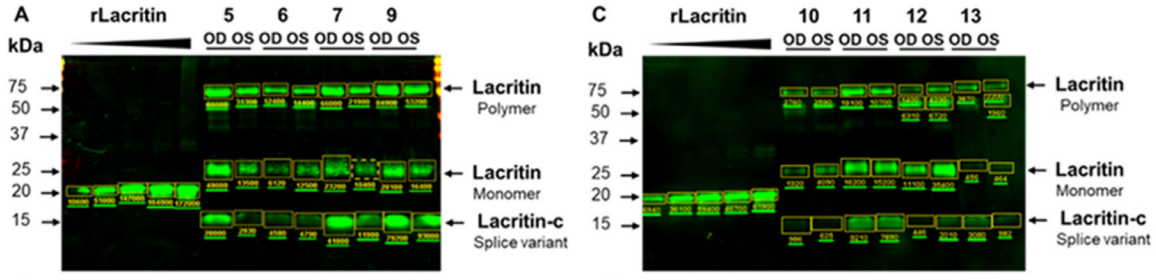
the same blot with fluorescent signals. Figure 6C is a summary table of the concentration data obtained from the blot. Percent proteoforms were calculated as nanograms proteoform per 100 ng total protein loaded in each lane. The micromolar concentration of each proteoform was calculated from the volume of tear sample/molecular mass (µL/µg) factoring in the molecular weight. The volume of tear samples loaded in each lane was approximated by the following equation: µL tears = mm (Schirmer value) + 0.07/1.34. Figure 7 is a composite of tear samples from individuals 5 through 20 shown with blots of the fluorescent signals for each proteoform and recombinant lacritin. Also shown are tables for each blot with a summary of the proteoform concentrations.

Distribution of Total Lacritin and Proteoforms in Tear Samples

Quantitation of each lacritin proteoform was determined in nanograms for the 40 tear samples with a linear regression equation derived from a standard curve of known recombinant lacritin concentrations run on the same blot as shown in the Figure 6 example. Percent proteoforms were calculated as nanograms proteoform per 100 ng total protein loaded in each lane.Figure 8 shows the percent lacritin-c, percent monmer, percent polymer proteoforms, and total lacritin as a box plot quartile distribution.

Discussion

The concentrations and relative distribution of proteins found in tear film can define a healthy homeostasis for the ocular surface, and the reduction of specific tear proteins has been associated with a variety of ocular diseases. Biochemical analysis of tear proteins from diseased individuals can provide biomarkers for the diagnosis of ocular diseases and enable development of new drugs for replacement therapies. The collection of tears is relatively noninvasive in a predominately cell-free environment. The use of Schirmer test strips is an established clinical procedure that provides a measurement of tear production in millimeters adsorbed for the diagnosis of dry eye. Can Schirmer test strip values also provide a measurement of tear volumes adsorbed? As shown

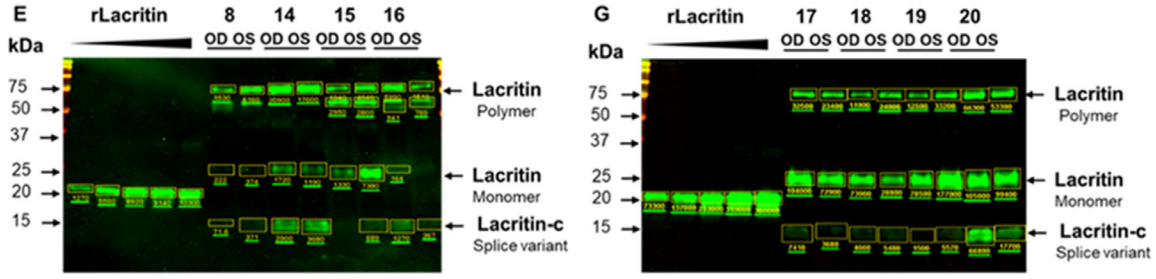


B

Sample Number	5	5	6	6	7	7	9	9
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
Schirmer (mm)	27	19	9	9	35	30	35	35
Protein (mg/mL)	2.8	2.1	1.0	1.3	1.9	1.4	2.3	1.8
ng Lacritin-c	65	38	40	40	81	48	122	137
% Lacritin-c	2.6	1.5	1.6	1.6	3.2	1.9	4.8	5.4
µM Lacritin-c	23	17	14	17	16	10	35	26
ng Monomer	88	49	41	49	61	47	67	53
% Monomer	3.4	1.9	1.6	1.9	2.4	1.8	2.6	2.1
µM Monomer	27	20	12	18	10	9	17	9
ng Polymer	108	69	48	50	107	59	128	93
% Polymer	4.2	2.7	1.9	2.0	4.2	2.3	5.0	3.6
µM Polymer	6	5	3	3	3	2	6	3

D

Sample Number	10	10	11	11	12	12	13	13
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
Schirmer (mm)	10	14	8	6	16	15	18	25
Protein (mg/mL)	1.3	1.8	1.3	1.3	1.9	1.9	2.0	2.1
ng Lacritin-c	ND	ND	57	52	ND	31	35	ND
% Lacritin-c	ND	ND	2.2	2.0	ND	1.2	1.4	ND
µM Lacritin-c	ND	ND	27	33	ND	17	17	ND
ng Monomer	31	39	81	78	63	149	ND	ND
% Monomer	1.2	1.5	3.2	3.0	2.5	5.8	ND	ND
µM Monomer	11	13	34	42	19	72	ND	ND
ng Polymer	34	33	92	62	ND	40	38	32
% Polymer	1.3	1.3	3.6	2.4	ND	1.5	1.5	1.3
µM Polymer	2	2	7	6	ND	3	3	2



F

Sample Number	8	8	14	14	15	15	16	16
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
Schirmer (mm)	5	5	30	35	7	6	19	10
Protein (mg/mL)	2.4	1.5	2.5	2.4	2.2	2.6	2.3	1.6
ng Lacritin-c	ND	ND	63	80	ND	13	26	ND
% Lacritin-c	ND	ND	2.4	3.1	ND	0.5	1.0	ND
µM Lacritin-c	ND	ND	20	23	ND	17	12	ND
ng Monomer	ND	ND	36	25	28	162	ND	ND
% Monomer	ND	ND	1.4	1.0	1.1	6.3	ND	ND
µM Monomer	ND	ND	10	6	26	176	ND	ND
ng Polymer	32	135	463	389	39	88	131	32
% Polymer	1.3	5.3	18.1	15.2	1.5	3.4	5.1	1.2
µM Polymer	7	20	23	17	7	17	9	3

H

Sample Number	17	17	18	18	19	19	20	20
OD/OS	OD	OS	OD	OS	OD	OS	OD	OS
Schirmer (mm)	12	10	24	15	5	7	35	25
Protein (mg/mL)	1.7	1.4	2.2	1.9	1.5	1.8	2.5	2.3
ng Lacritin-c	8	5	6	6	ND	6	45	14
% Lacritin-c	0.3	0.2	0.2	0.3	ND	0.3	1.7	0.5
µM Lacritin-c	3	2	2	2	ND	5	13	5
ng Monomer	68	48	49	21	52	113	69	65
% Monomer	2.6	1.9	1.9	0.8	2.0	4.4	2.7	2.5
µM Monomer	26	17	16	7	44	71	17	21
ng Polymer	23	17	10	18	11	24	44	36
% Polymer	0.9	0.7	0.4	0.7	0.4	0.9	1.7	1.4
µM Polymer	2	1	1	1	2	3	2	2

Figure 7. Quantitation of lacritin proteoforms from 16 individuals. Figure shows fluorescent signals generated for each lacritin proteoform from individuals 5 through 20 and a standard curve of signal versus known concentrations of recombinant lacritin. Also shown are summary tables of data generated from each set of eight tear samples obtained from both eyes (OD and OS) of four individuals (percent = ng lacritin proteoform/100 ng total protein). ND, not detected.

in Figure 1, when increasing volumes (in µL) of protein or contrived tears were adsorbed to Schirmer test strips, there was a strong correlation to the Schirmer values measured (R^2 = 0.99). From these data, the volume of tears collected from Schirmer test strips can be approximated by the equation µL tears = mm (Schirmer value) + 0.07/1.34, which can be used to calculate micromolar concentrations of tear proteins of known molecular mass (ng) and molecular weight (ng/µL). An important consideration in using Schirmer test strips for the collection of tear proteins is elution and recovery of proteins and in particular recovery

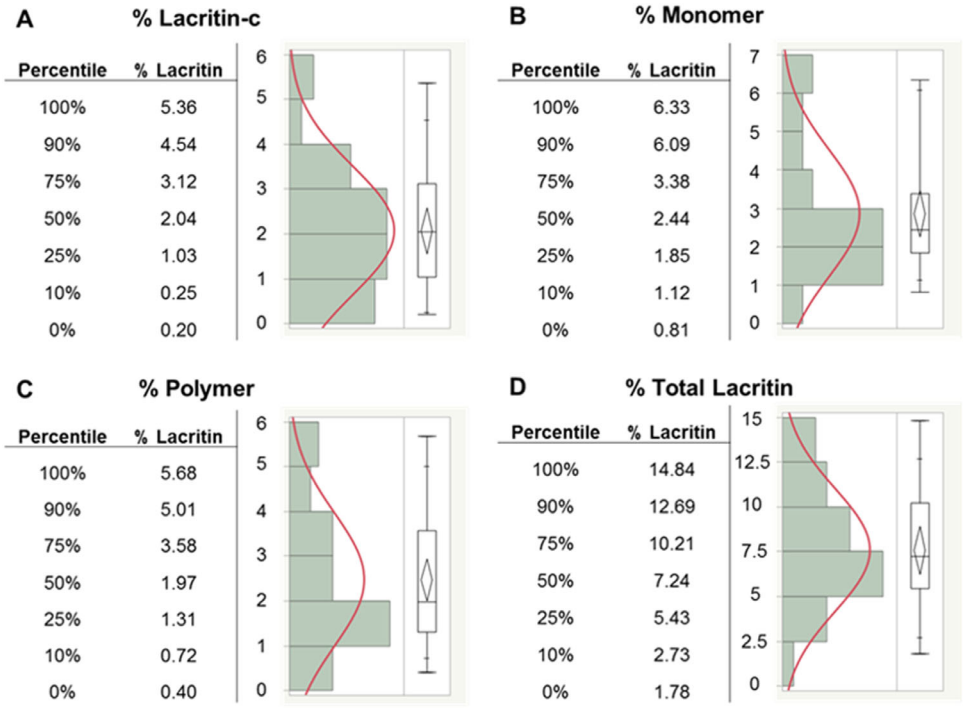


Figure 8. Distribution of lacritin proteoforms in 40 tear samples. Box plot quartile distributions of each lacritin proteoform are presented as percent total protein (percent = ng lacritin proteoform/100 ng total protein). The sum of all lacritin proteoforms is presented as percent total lacritin: (A) percent lacritin-c, (B) percent monomer, (C) percent polymer, and (D) percent total lacritin.

of the proteins to be analyzed. Figure 2 shows that proteins adsorbed to Schirmer test strips eluted by diffusion and centrifugation were recovered in excess of 90% compared to proteins not adsorbed/desorbed (100%). Also shown in Figure 2 is a spiking experiment that quantitates recovery of recombinant lacritin from the Schirmer test strips.

A number of methods have been reported for the biochemical analysis of tear proteins recovered from Schirmer test strips. Here, we report the development of a quantitative immunoassay based on western blot analysis. Anti-lacritin antibodies detect three distinct proteoforms of lacritin in human tears. A full-length mature lacritin termed "monomer" has been shown to exhibit pro-tearing and pro-ocular surface homeostasis activities, and it is selectively deficient in most dry eye tears. An inactive tissue transglutaminase cross-linked polymer has been shown to be a negative regulator of monomeric lacritin bioactivity.[30] A splice variant of lacritin with unique intron coding sequences and unknown functions, termed lacritin-c, has also been detected in human tear samples. As shown in Figure 3, anti-Pep Lac N-term antibodies detects all three proteoforms of lacritin, as well as purified recombinant lacritin, whereas antibodies unique to intron 3 of lacritin detect only lacritin-c and polymers of lacritin-c by western blot analysis. It is interesting to note that lacritin-c does not contain C-terminal coding sequences shown to be the active site of lacritin[22]; however, it is detected in most of the tear samples analyzed. It is also detected as a polymer that may be crossed-linked with itself or with monomeric lacritin, perhaps also acting as a negative regulator of lacritin bioactivity.

To validate the assay, tear samples were collected from both eyes of 20 healthy individuals and eluted from Schirmer test strips, and the total protein content was determined. Figure 4 shows a scatterplot of eluted protein concentrations that ranged from 1.0 to 3.1 mg/mL. Figure 5 provides a composite of five western blots of 40 tear samples collected from both eyes of 20 individuals challenged with anti-Pep Lac N-term antibodies showing the three distinct lacritin proteoforms. These same blots were quantified by the fluorescent signals generated by the LI-COR Odyssey CLx for each of the three proteoforms of lacritin. In order to determine nanogram values for each proteoform, a standard curve of signal versus known concentrations of recombinant lacritin was run on each blot to generate a linear regression equation for calculations of nanogram values for each lacritin proteoform as shown in Figure 6 for the first eight tear

samples collected from four individuals. Figure 7 is a composite of four western blots showing quantitation of the remaining 32 samples from 16 individuals. Tables associated with each western blot summarize the data generated. In addition to Schirmer values (mm) and total protein concentrations (mg/mL) for each sample, concentrations for each lacritin proteoform can be calculated. From the nanogram values generated by the standard curve, micromolar concentrations were determined using the equation µL tears = mm (Schirmer value) + 0.07/1.34, as well as the molecular weight (ng/µL). The percent total protein for each proteoform was calculated as percent = ng proteoform/100 ng total protein loaded on the western blot.

Although the relatively small sample size of 40 samples from 20 individuals with no history of ocular diseases who are otherwise healthy does not allow a statistically significant extrapolation to draw conclusions about a healthy or normal population at large, some observations can be discussed regarding the distribution of total protein and lacritin concentrations in tear samples as shown in Figure 8. The median concentration of eluted proteins was 1.9 mg/mL with a distribution from 1.0 to 3.1 mg/mL. Total nanogram lacritin values ranged from 45 to 562, with a median of 211, and the median percent total lacritin (ng lacritin/100 ng total protein) was 7.2%, with a distribution between 1.8% and 14.8%. The relative distributions of the different proteoforms of lacritin are also shown. Here, we have reported a methodology for human tear collection, processing, and separation and the quantitative analysis of tear lacritin proteoforms validated with tears from 20 adults in preparation for analysis of tear samples from the phase II clinical trial of Lacripep in subjects with dry eye associated with primary Sjögren's syndrome.

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