

ORIGINAL ARTICLE

Validation of the Flush Method as an Alternative to Basal or Reflex Tear Collection

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ABSTRACT

Purpose: To validate the more easily applicable “flush” tear collection technique as a viable alternative to basal and reflex tear collection.

Materials and Methods: Total protein content (TPC) and immunoglobulin A (IgA) concentrations were determined in the basal, reflex, and flush tears of 16 healthy non-contact lens wearers. The overall protein profile was established using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (MS).

Results: Collection-rates were 4.6 ± 6.7 $\mu\text{L}/\text{min}$, 13.9 ± 11.1 $\mu\text{L}/\text{min}$, and 25.7 ± 12.4 $\mu\text{L}/\text{min}$ for the basal, reflex, and flush tears, respectively. TPC was 7.14 ± 2.22 mg/mL , 6.01 ± 2.11 mg/mL , and 3.79 ± 1.51 mg/mL for basal, reflex, and flush tears, respectively, with flush tears being significantly less concentrated than basal ($p=0.001$) and reflex ($p=0.008$). IgA concentration was 1.04 ± 0.29 mg/mL , 0.64 ± 0.26 mg/mL , and 0.65 ± 0.23 mg/mL for basal, reflex, and flush tears, respectively, with basal tears being significantly more concentrated ($p<0.001$). As a percentage of TPC, IgA represented $19.8 \pm 14.9\%$, $11.4 \pm 3.9\%$, and $19.8 \pm 8.7\%$ for basal, reflex, and flush, respectively. The flush was not significantly different to basal ($p=1.00$) but significantly greater than reflex ($p=0.02$). SDS-PAGE showed the same tear profiles for basal and flush tears. MS identified the most abundant proteins in all tear types.

Conclusions: The flush method allows much faster collection than basal secretion sampling but returns essentially the same spectrum of proteins in similar proportions. This behavior is confirmation that the flush technique has utility as a more convenient alternative to basal tear sampling in studies involving composition analysis.

KEYWORDS: Basal tears; Flush tears; Human tear film; Reflex tears; Tear collection; Tear proteins

INTRODUCTION

As the pre-ocular tear film is critical to the integrity of the ocular surface, assessment of its biochemical characteristics presents a valuable means of studying this region of the eye in both health and disease. Examples include the use of tear fluid analysis to determine changes contributing to altered corneal integrity in keratoconus¹ and diabetes,² establish differences

between the open and closed-eye environments,³ and assess the effect of contact lens wear on the ocular surface.^{4–8}

Tear samples can be obtained from human subjects in several ways, but drawbacks are associated with each approach. For example, tears can be absorbed into Schirmer strips resting on the lower eyelid and subsequently eluted for biochemical analysis.^{9–11} Although simple to perform, this procedure tends to collect cellular, as well as secreted, proteins, and the physical presence of the strip can cause mechanical stimulation of the corneal and conjunctival epithelium. As a result, the composition of the collected sample may differ from that in pure basal secretion.^{12–14}

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A common alternative is to use microcapillary tubes to draw tears from the reservoir within the conjunctival sac, usually at the fornices. While generally less invasive than Schirmer strips, attempts to collect basal tears with this method can be tedious, particularly where there is a paucity of flow, as often occurs in dry eye or when the eyes are opened after awakening. To overcome this, it is often necessary to pool samples as a means of generating adequate volume for analysis.^{9,15} When collecting basal tears, it is also challenging to avoid inadvertent contact with the lids, and/or ocular surface, and thus, induce reflex tearing.

An option is to simply provoke or utilize reflex tearing as a means of providing increased tear volume at a faster rate. This is itself an inherently variable endeavor that is difficult to perform with consistency between collection attempts. While actual collection rates may be relatively steady on any given occasion, the rate of tear production is not amenable to effective control.¹⁶ In addition to volumetric changes, the procedure of inducing reflex tears also influences the spread of proteins in the sample. Although the main regulated proteins of the lacrimal gland, such as lysozyme, lactoferrin, and tear lipocalin remain constant, others such as immunoglobulin A and G, albumin, and fibronectin change dramatically.^{16,17}

A number of workers^{18–26} have attempted to make the collection process less demanding by sampling tears after first flooding the eye with saline. The idea is that the increased fluid volume will be much easier, and quicker, to coax into a micro-capillary tube but will still contain the same moieties as normal tears, albeit at a reduced concentration.

This assumption has only been partially verified. One-dimensional gel electrophoresis has shown that similar major proteins appear in samples collected by the “flush” method as are in basal tears,¹⁹ but total protein measures appear to be more variable when compared to reflex tears.^{21,22} Part of the reason for this may be that the technique used in these studies involved multiple instillations of saline. In addition to sample dilution, this might well have triggered reflex tearing and, therefore, contributed to the variability.

It is also noteworthy that the technique itself is not applied in any standard fashion. Among the studies conducted to date, both the number and volume of saline installations have varied among the various groups reporting on its use.^{18–24,26} These technical issues cast doubt on the validity of the flush method as a means of providing tear samples that are truly representative of the fluid existing within the conjunctival sac.²⁷ Additionally, the behavior of the less abundant protein species in the flush tear collection is not known.

With this in mind, we proposed a standard method of flush tear collection based on the single application of a controlled volume of saline. The purpose of the present article is to describe both the technique, and our efforts to validate it by establishing test-retest repeatability, and comparing the amount and variety of proteins contained in flush samples with those in conventionally obtained basal and reflex secretions. Both major and less abundant protein species were evaluated to give as broad a picture as possible of differences between the techniques.

MATERIALS AND METHODS

Study Participants

This study was approved by the Institutional Ethics Committee of the University of New South Wales. Informed consent was obtained from 16 non contact lens wearers (11 female, 5 male) according to the tenets of the Declaration of Helsinki. All participants were aged between 18 and 45 years of age. Participants were healthy and were not taking medications for any general health conditions.

Study Design

A total of six tear samples were collected from all participants at the Brien Holden Vision Institute by the same experienced investigator, (MM). Each of the basal, reflex, and flush methods described below was used on two occasions in random order allowing at least 24 hr between collections, so that tears were collected six times in total for each participant. At each visit, tears were obtained from both eyes and were not pooled, the right eye always being collected first. A new capillary was used for each eye. Collection times ranged between 10:45 am and 16:35 pm; however, the majority were between 12:00 pm and 2:00 pm.

Tear Collection Method

Basal Tear Collection

Basal tears were collected using a 10 μ L microcapillary tube (Blaubrand® intraMark, Wertheim, Germany). The collection time was limited to a maximum of 2 min, which was deemed long enough to enable adequate collection, but short enough to avoid stimulation of reflex tears.²⁸ Participants tilted their head towards the side of collection while looking in the opposite direction. The microcapillary tube rested in the lateral tear meniscus. Care was taken to minimize contact

with the bulbar conjunctiva or the lid margin to avoid the risk of stimulation. Participants were allowed to blink during the procedure, after first withdrawing the microcapillary tube and collection was paused if any reflex tearing was suspected. A fresh capillary tube was used for each eye. The tear collection rate was determined by noting the sampling time and volume collected. In circumstances where persistent reflex tearing was evident, the procedure ceased and the collection rate was calculated based on the time elapsed to that point.

The sample was expelled from the capillary tube into a siliconized polypropylene microcentrifuge tube of 0.65 mL capacity (Sigma-Aldrich, Steinheim, Germany) and placed on ice until processing.

Reflex Tear Collection

Reflex tears were collected as for basal tears except that the sneeze reflex was stimulated by gently inserting a sterile cotton bud into the nasal passages to induce tear production. Collection took place for approximately 1 min to enable sufficient volume to be obtained.

Flush Tear Collection

Flush tears were collected by instilling a single, 60 μ L drop of non-preserved, unit dose saline (Sodium Chloride injection 0.9%, AstraZeneca, North Ryde, Australia) into the inferior palpebral fold using an Eppendorf pipette. Care was taken not to touch the eye with the pipette tip. Participants were then instructed to gently close their eyes and rotate them twice. Fluid was then immediately collected by capillary as before, with a limit of 1 min being imposed to avoid reflex tearing.²⁸

Treatment of Tear Samples

After collection, samples were centrifuged at 4000 rpm force for 20 min at 4°C to remove cellular debris. The supernatants were collected and stored in two aliquots in siliconized polypropylene microcentrifuge tubes (Sigma-Aldrich) at -80°C. One aliquot was used for total protein content and the other for immunoglobulin A and any further analysis. The samples were analyzed within one month of tear collection. No protein extraction solvent was applied prior to running the samples.

Total Protein Content

Total protein content (TPC) was determined using the bicinchoninic acid method (BCA) and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester,

New York, USA) following the manufacturer's instructions.

Immunoglobulin A Analysis

Immunoglobulin A (IgA) concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the Bethyl Human IgA Quantitation kit (Montgomery, Texas, USA) following the manufacturer's instructions. Wells of Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific) were coated with 100 μ L of the primary antibody. The antibody was allowed to bind overnight at room temperature for the first eight participants and for 3 hr for the second group of eight participants. The plates were washed three times with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween20, pH 8.0). The wells were then blocked with 200 μ L blocking solution containing 50 mM Tris, 0.14 M NaCl, 1% BSA (pH 8.0) for 30 min. The blocking solution was then removed and the wells were washed three times with the wash buffer. A volume of 100 μ L of tears diluted in sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) were loaded at 1:3000 dilutions in duplicate. These were allowed to incubate for 1 hr and the wells were then washed five times with the wash buffer. A volume of 100 μ L of horseradish peroxidase (HRP) detection antibody was added to each well in a dilution of 1:20,000. This was incubated for 1 hr after which it was removed and the wells were aspirated with wash buffer. Tetramethylbenzidine (TMB) peroxidase substrate was added to each well in a volume of 100 μ L and stopped with 2 M sulfuric acid (H_2SO_4) after 30-min incubation. The optical density was then measured at 450 nm. Each plate included seven dilutions of the standard in duplicate from which the standard curve was generated. The average absorbance of the blank wells was subtracted from the average of each sample and each standard in order to determine the final absorbance.

Tear Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Tear SDS-PAGE followed by scanning densitometry was performed as a means of qualitative analysis of the major tear proteins of the basal and flush tears of five participants. SDS-PAGE analysis was performed on the Bio-Rad Mini-Protein® Tetra Cell (Bio-Rad, Hercules, California, USA) in a Laemmli discontinuous buffer system.²⁹ Tear samples were mixed with SDS PAGE loading buffer (0.5 M Tris-HCL, pH 6.8, 10% SDS, 0.5% Bromophenol Blue, glycerol, β -mercaptoethanol) and denatured by heating at

100°C for 5 min. A fixed mass of 10 µg was added to each well as calculated from the TPC. Tear proteins were separated using a homogenous 12% gel (40% acrylamide/Bis, 1.5 M Tris-HCL pH 8.8, 10% SDS, TEMED, 10% ammonium persulfate) and a 4% stacking gel (40% acrylamide/Bis, 0.5 M Tris-HCL 6.8, 10% SDS, TEMED, 10% ammonium persulfate) cast in-laboratory. Upon loading, the tear samples were run at 200 V for approximately 1 hr in a running buffer (Tris base, glycine, 10% SDS, pH 8.3). A Precision Plus Protein™ Standard (Bio-Rad, Hercules, California, USA) was run in parallel to the samples as a marker. Gels were fixed and then stained with Coomassie blue R250 and were imaged using the Bio-Rad GS 800 scanner (Bio-Rad). Lane profiles were analyzed using Quantity One software (Bio-Rad). One gel was performed for each participant.

Mass Spectrometry (MS)

Liquid chromatography mass spectrometry (MS) was conducted on three replicates of each sample type, all from the same participant, in order to gauge whether the same spectrum of proteins was identifiable across tear collection methods. A 2 µl volume of a 1:5 dilution of DTT to ammonium bicarbonate (NH_4HCO_3) was added to a 10 µg solution of the tear sample, calculated based on TPC. This was denatured for 20 min. An additional reducing agent, IA 200 mmol, was added in a 1:5 dilution in NH_4HCO_3 . A 2.1 µl volume was added to each sample and further denatured for 20 min. The sample was digested with trypsin, where trypsin was diluted in NH_4HCO_3 in a 1:2.5 dilution. This was denatured overnight. The following morning each of the digested samples were diluted in 1% formic acid (FA) and 0.05% heptafluorobutyric acid (HFBA) in 1:25. The digest was then loaded onto a strong cation ion exchange column (SCX). The fractions from the SCX chromatograph were collected and further separated using a reverse phase C18 column which was coupled with an ESI mass spectrometer.

All MS/MS samples were analyzed using Mascot software (Matrix Science, London, UK; version Mascot).³⁰ Mascot was set up to search the National Center for Biotechnology Information (NCBI) nr_19_1_09 database (unknown version, 7686184 entries) using the digestion enzyme trypsin. Mascot was searched with a fragment ion (MS/MS) mass tolerance of 0.40 Da and a parent ion (MS) tolerance of 4.0 ppm. Oxidation of methionine, iodoacetamide derivative of cysteine, acrylamide of unknown, and acrylamide adduct of cysteine were specified in Mascot as variable modifications. Scaffold (version Scaffold_2_04_00, Proteome Software Inc., Portland,

Oregon, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm.³¹ Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.³² Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Data Analysis

Repeated measures analysis of variance (ANOVA), with Bonferroni corrected Student's *t*-tests post-hoc, was used to compare the three collection methods (*p*-value < 0.05 denotes statistical significance, SPSS 16.0 GP). Missing data were replaced using an expectation maximization (EM) procedure, substituting zeros when negative values were generated. Sphericity was verified with Mauchly's test.

RESULTS

Tear Collection

There were no significant differences between right and left eyes (*p*=0.38), collection days (*p*=0.36), or gender (*p*=0.45) in terms of collection rate for any of the methods.

Accordingly, data were averaged across both eyes and days for each participant before conducting further analysis. Table 1 shows the group mean collected volume and collection rate for each method.

Both volume and collection rate were highest for the flush tears. All the collection rates were significantly different between methods (*p*<0.05).

Day to Day Repeatability

Repeated measures ANOVA showed no difference between eyes or days for any of the methods, whether

TABLE 1 Group mean ± standard deviation of collected volumes and collection rates based on standard collection times of 2 min for basal and 1 min for reflex and flush (*n*=16)

Collection method	Volume (µL ± SD)	Collection rate (µL/min ± SD)
Basal	8.6 ± 11.8	4.6 ± 6.7
Reflex	14.6 ± 11.6	13.9 ± 11.1
Flush	27.2 ± 11.7	25.7 ± 12.4

for TPC ($p=0.13$ and 0.22 , eyes and days, respectively), IgA concentration ($p=0.07$ and 0.40), or their ratio ($p=0.77$ and 0.13).

Coefficients of repeatability (COR)³³ are shown in Table 2 along with the Limits of Agreement.

TPC and IgA Analysis

As there were no significant differences between eyes or days for either TPC or IgA, data were averaged prior to comparison between collection methods. Table 3 summarizes the outcome and it can be seen that, as expected, TPC was highest for basal and least for flush tears. The latter were significantly less concentrated than either basal or reflex tears ($p=0.001$ and 0.008 , respectively).

IgA concentration in basal tears was significantly greater than in both reflex ($p=0.002$) and flush tears ($p<0.001$).

However, when expressed as a percentage of TPC, the amount of IgA was identical in basal and flush samples.

SDS-PAGE

Figure 1 shows the SDS-PAGE patterns for basal and flush tears (representative) from one participant. Only basal and flush tears were compared as these were found to be proportionately similar in the previous protein analysis. Lane profile analysis exhibited the presence of all major protein bands in both basal and flush tear collection methods, the molecular

weight (MW) of these being consistent with those known for lactoferrin, albumin, IgA, lipocalin, and lysozyme.³⁴ The gel band intensities were profiled in order to identify regions of differences. In each case the same peaks were present for both basal and flush samples.

Mass Spectrometry (MS)

In order to give as broad a picture of the less abundant proteins in tear samples, not identifiable by SDS-PAGE, MS was performed on three replicates from each tear collection method, all from the same participant, according to the aforementioned protocol. The results are shown in Table 4. As a means of showing commonality between the three replicates for each method, Venn diagrams were constructed as shown in Figure 2. From these it can be seen that basal sampling was the most consistent as 26 out of the total 29 proteins detected were present in all three replicates. Consistency was slightly reduced for both the flush and reflex methods, which repeatedly returned 18 out of 27 and 18 out of 29 proteins, respectively. Taking only the consistently returned proteins for each method, we constructed a further Venn diagram to examine the distribution of these moieties between techniques. This is shown in Figure 3 where it can be seen that 15 proteins were common to all three sampling groups. These 15 are the more abundant proteins and are indicated with asterisks in Table 4. Comparing only flush and basal collection showed 17 common proteins, the same number as did reflex and basal.

TABLE 2 Limits of agreement (mean $\pm$ two standard deviations of the differences) and coefficients of repeatability ($1.96 \times$ the standard deviation of mean difference between replicates) for each tear collection method for total protein content (TPC) and immunoglobulin A (IgA)

			Basal	Reflex	Flush
Limits of agreement	TPC (mg/ml)	Days	-3.19 to 7.55	-7.49 to 6.97	-3.49 to 4.15
		Eyes	-6.35 to 5.61	-7.66 to 6.22	-3.97 to 3.05
	IgA (mg/ml)	Days	-0.46 to 0.24	-0.48 to 0.44	-0.36 to 0.33
		Eyes	-0.35 to 0.61	-0.39 to 0.35	-0.12 to 0.38
Coefficient of repeatability	TPC (mg/ml)	Days	5.3	7.2	3.8
		Eyes	6.0	6.9	3.5
	IgA (mg/ml)	Days	0.58	0.93	0.69
		Eyes	0.68	0.75	0.49

TABLE 3 Group mean ($\pm$ standard deviation) total protein content (TPC), immunoglobulin A (IgA) concentration, and IgA as a percentage of TPC for basal, reflex, and flush tears ($n=16$) with associated p -values

	Basal	Reflex	Flush	p -Value		
				B-R	R-F	B-F
TPC (mg/ml)	7.14 ± 2.22	6.01 ± 2.11	3.79 ± 1.51	0.15	0.001	0.008
IgA concentration (mg/ml)	1.04 ± 0.29	0.64 ± 0.26	0.65 ± 0.23	< 0.001	1.00	0.002
IgA percentage (%)	19.8 ± 14.9	11.4 ± 3.9	19.8 ± 8.7	0.15	0.02	1.00

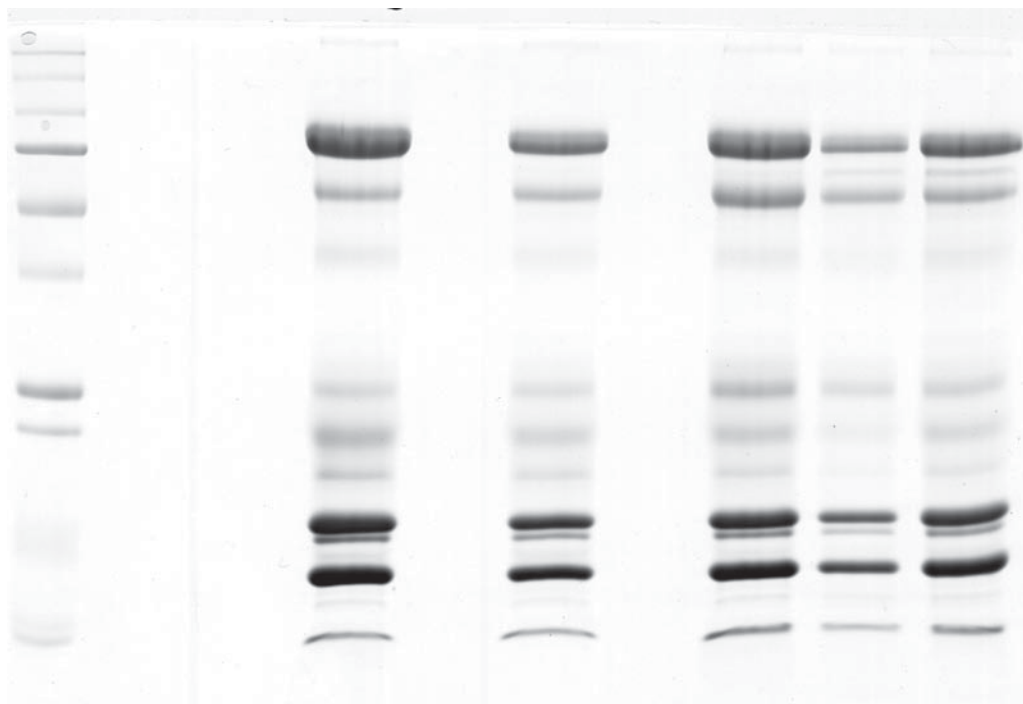


FIGURE 1 Representative SDS-PAGE gel of Coomassie-stained proteins in basal and flush tears for a single participant. Lane one is the molecular weight marker (MW) and each of the five subsequent lanes includes 10 µg of protein load.

DISCUSSION

When performing tear collection, the aim is to obtain a true representation of the status of the tear film at that point in time. In conducting the present study we wanted to establish if a standardized flush method of tear collection would permit such a view to be obtained more easily than the traditional approach of collecting basal secretions, while maintaining adequate repeatability between replicate events.

The rapidity of the flush technique is obvious from Table 1, where it can be seen that increasing the volume of fluid in the conjunctival sac allows a standard 10 µL sample to be collected in less than 30 sec, on average. This is at least four times faster than traditional basal sampling and, even taking into account the need for an additional saline instillation step, provides a less challenging and more comfortable procedure for subjects and investigators. The slower than expected collection rate in the reflex tear collection suggests that the single stimulus used here, triggering the “sneeze reflex,” is not always consistent in initiating reflex tearing. This supports the use of more than one method by some authors in order to facilitate this process.³⁵ Our faster than expected flow rate in the basal tear collection suggests that this method was not always consistent in collecting only basal tears.

Of course, speed is of no value unless the contents of the sample maintain fidelity with those of the original which, in this instance, are best represented by basal secretions. To evaluate the extent to which this was achieved, we compared our flush samples with basal tears from the same subjects, using a number of indicators.

SDS-PAGE was used to show that the capture of major proteins was essentially the same between the two methods (Figure 1). To add further weight to these results, we also looked at the status of a wider range of protein moieties using mass spectrometry. The Venn diagrams in Figure 2 show that consistency of proteins collected between replicates was similar for reflex and flush, but both were reduced relative to the basal sampling method. Thus, where basal sampling consistently returned 26 proteins, reflex and flush produced only 18. However, as can be seen from Figure 3, 15 of these consistent proteins were common to all the techniques and these were identified as being the more abundant species (asterisked in Table 4). It should be noted that basal tears returned 7 species not regularly found by the reflex or flush methods. Basal tears, therefore, appear to consistently collect a greater number of the less abundant proteins compared to reflex and flush tears. Reflex and flush essentially return the same proteins.

TABLE 4 Proteins in the basal, reflex, and flush tears of one participant as identified by mass spectrometry

Identified proteins (32)	Accession number	Molecular weight	Flush replicates			Reflex replicates			Basal replicates		
			1	2	3	1	2	3	1	2	3
			Percent coverage (percentage of peptides of that protein found)								
Lactoferrin*	gi 186833	78 kDa	54%	56%	54%	56%	42%	60%	51%	53%	65%
Lipocalin*	gi 4504963	19 kDa	65%	62%	62%	62%	52%	62%	62%	62%	62%
The complex formed between Mhc-like zinc alpha2-glycoprotein and prolactin inducible protein*	gi 145579641	14 kDa	70%	70%	75%	70%	44%	75%	81%	70%	75%
Mutant human lysozyme substituted at the surface positions*	gi 12084272	15 kDa	55%	65%	58%	71%	58%	64%	64%	65%	60%
Unnamed protein product [Homo sapiens]*	gi 34527233	53 kDa	9%	14%	13%	15%	15%	11%	15%	18%	15%
PolymERIC immunoglobulin receptor*	gi 238236	83 kDa	11%	8%	9%	10%	9%	10%	27%	24%	16%
Cystatin S*	gi 4503109	16 kDa	54%	49%	41%	62%	20%	62%	49%	62%	49%
Proline rich 4*	gi 1050983	15 kDa	12%	28%	22%	22%	22%	28%	22%	22%	22%
Lactritin precursor*	gi 15187164	14 kDa	17%	30%	24%	30%	30%	23%	24%	24%	24%
Secretoglobulin*	gi 4505171	11 kDa	40%	32%	32%	59%	32%	59%	68%	59%	68%
Proline rich, lacrima 1*	gi 116805344	27 kDa	12%	12%	12%	12%	12%	12%	12%	7%	12%
Unnamed protein product [Homo sapiens]	gi 189053131	19 kDa	39%	44%	44%	26%	0%	39%	39%	39%	39%
Immunoglobulin J chain*	gi 114319027	20 kDa	15%	11%	15%	27%	23%	15%	33%	27%	23%
Apolipoprotein	gi 178855	49 kDa	15%	7%	0%	7%	0%	7%	21%	13%	17%
Lipophilin A*	gi 5729907	10 kDa	10%	10%	10%	10%	10%	22%	23%	22%	10%
Proline rich 4 (lacrima) isoform 1	gi 154448882	17 kDa	0%	10%	10%	10%	10%	10%	10%	10%	10%
Ig alpha-2 chain C region*	gi 70058	37 kDa	14%	4%	4%	14%	14%	14%	4%	4%	14%
Contribution of hydrophobic residues to the stability of human lysozyme*	gi 157832585	15 kDa	22%	22%	22%	22%	22%	22%	22%	22%	22%
Ig L-chain V-region	gi 27552515	23 kDa	7%	7%	0%	7%	7%	0%	20%	20%	7%
Ig kappa chain V-III (KAU cold agglutinin)	gi 106586	23 kDa	9%	0%	0%	9%	0%	9%	18%	26%	9%
Solution structure of W60g mutant of human beta2-microglobulin	gi 158431179	12 kDa	10%	20%	10%	10%	0%	10%	0%	0%	0%
structural changes of the active site cleft and different saccharide binding modes in human lysozyme	gi 1065033	15 kDa	22%	0%	22%	0%	0%	22%	22%	0%	22%
Human tear lipocalin von Ebners gland protein	gi 56554584	18 kDa	10%	10%	10%	10%	0%	10%	10%	10%	10%
Ig Aalpha1 Bur	gi 223099	51 kDa	5%	0%	5%	5%	5%	5%	5%	5%	5%
Ig A H	gi 229537	51 kDa	0%	0%	0%	1%	1%	1%	1%	3%	0%
Phospholipase A2	gi 119615316	12 kDa	0%	0%	0%	0%	0%	9%	26%	9%	26%
Unnamed protein product [Homo sapiens]	gi 194384278	33 kDa	4%	4%	0%	4%	0%	4%	4%	4%	4%
Deleted in malignant brain tumors 1, isoform CRA_a	gi 119569693	112 kDa	1%	1%	0%	1%	0%	1%	0%	1%	1%
Mesothelin or CAK1 antigen precursor	gi 1145724	69 kDa	0%	0%	0%	0%	0%	0%	4%	0%	0%
Immunoglobulin alpha heavy chain variable region	gi 62871224	14 kDa	0%	0%	14%	0%	0%	0%	14%	14%	14%
Secretory Iga1	gi 146387599	64 kDa	0%	0%	0%	0%	0%	0%	5%	5%	5%
Human cystatin C	gi 14278690	13 kDa	0%	0%	0%	0%	0%	22%	0%	0%	0%

Asterisks (*) indicate proteins captured in all replicates and collection methods. Gray boxes highlight the proteins not identified in that sample.

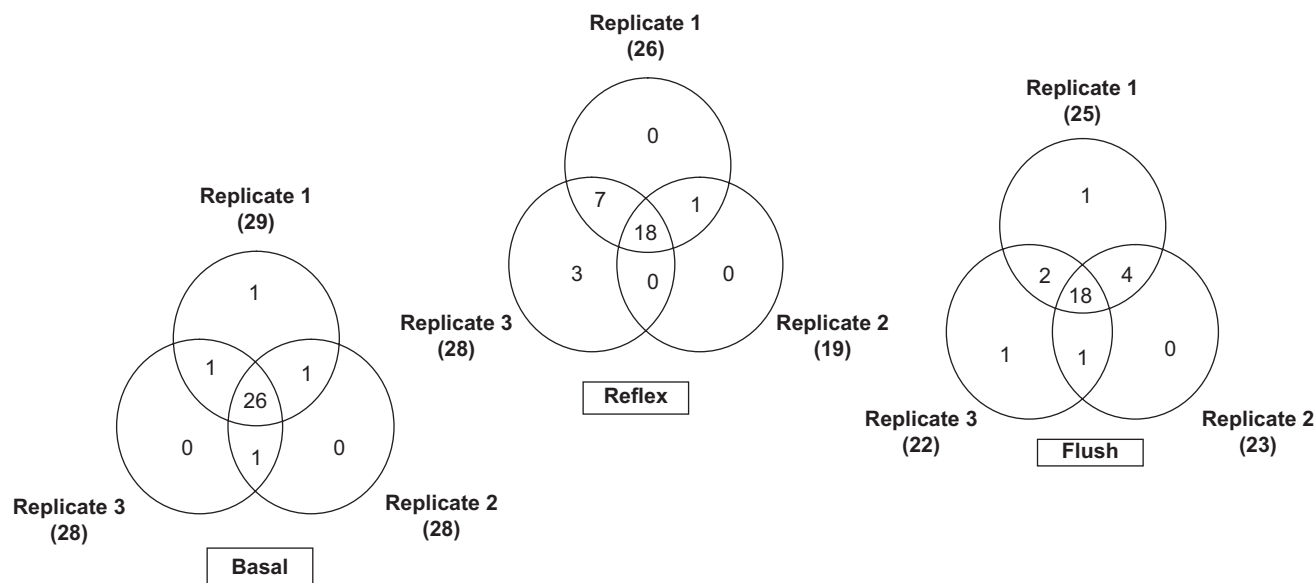


FIGURE 2 Venn diagrams indicating commonality of protein species among three replicates each of basal, reflex, and flush tear samples. The number in brackets indicates the total number of proteins found in that replicate. Replicates 1 and 2 were taken on the same day, from the right and left eyes, respectively, while replicate 3 was taken from the right eye on the second day.

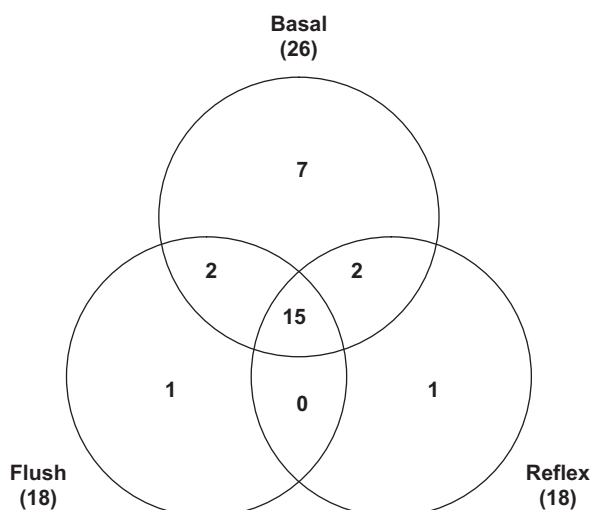


FIGURE 3 Venn diagram showing commonality among those proteins consistently captured by each collection method (Basal, Reflex and Flush). The numbers in brackets indicate the total number of proteins consistently captured in each of the three replicates of the same sampling method.

To investigate the behavior of protein concentration, we looked at both total protein and IgA. The latter was chosen as a representative protein because its concentration is known to alter as a result of ocular stimulation,³⁵ making it a reasonable indicator of the potential for flush methods to be invasive, relative to basal collection. Both total protein and IgA concentrations were reduced in the flush samples, as would be expected due to the increased volume. However, their relative amount was the same in flush and basal samples, as was indicated

by the IgA:TPC ratio (Table 3). Taking this observation together with those discussed earlier, provides good evidence that flush collection produces a sample that, while of low absolute concentration, largely mirrors basal tears both in terms of the most abundant proteins captured and their relative amounts. Future studies comparing the three techniques could examine the effect of saline instillation on proteins influenced by osmolarity³⁶ as well as the effect on concentrations of other proteins, such as albumin and inflammatory proteins.

Turning finally to repeatability, various investigators have reported high levels of variability when using the flush approach.^{21,22} From our perspective, this performance has probably been influenced by a lack of consistent methodology. In particular, the use of different numbers of multiple saline instillations between studies and even sampling events^{19,21,22} is far from ideal. Using a single, controlled volume of saline appears to largely solve this problem as, in terms of both total protein and IgA concentrations, repeatability for the flush method in the current study was at least as good as that for basal collection, and better than for reflex tears.

In summary then, we propose that in cases where less abundant proteins, that is, those not identifiable with SDS-PAGE, need to be identified consistently across samples, basal sampling remains the most suitable approach, as it collects a larger range of such moieties. In most other circumstances, however, the standardized method of flush tear collection outlined above can be considered as an alternative to conventional capillary tube tear sampling. It permits repeatable retrieval of largely the same range of the more abundant proteins,

in the same proportions as basal tears, but is quicker, and thus, less disturbing for subjects and more convenient for investigators.

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