

# Establishment of a Tear Ferning Test Protocol in the Mouse Model

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**Purpose:** Analysis of ferning formation after tear drop desiccation on a glass slide has been applied as a simple method to examine tear normality and is referred to as the tear ferning (TF) test. Despite use of the TF test in clinical settings and in some animals, thus far no TF test protocol has been developed for the mouse model. This study aimed to establish a mouse TF test protocol that can be used for dry eye research using the mouse as the study model.

**Methods:** Tear samples were collected from 24 healthy mice after repeated flushes with 2, 5, 10, or 20  $\mu$ L wash solutions, either 0.9% NaCl saline or sterile water, on the ocular surface. After sample collection, TF tests were performed at variable drop volumes (2–20  $\mu$ L), at a relative humidity of either  $46\% \pm 2\%$  or  $53\% \pm 2\%$ , and with temperature fixed at  $24^\circ\text{C} \pm 2^\circ\text{C}$  for comparison. Moreover, the influence of osmolarity (between 280 and 360 mOsm/L) and pH values (6.5–8.0) and the effect of centrifugation (4000 rpm, 10 minutes) on ferning formation were examined. Reproducibility and ferning storage stability were also determined.

**Results:** An optimized protocol was established with relative humidity at  $46\% \pm 2\%$  and drop aliquot at 2  $\mu$ L, using 0.9% NaCl saline as the wash solution. Using sterilized water as the wash solution did not result in any crystalloid formation. Centrifugation did not aid ferning formation in any of the samples. Higher osmolarity increased ferning formation from grades between 0 to 1 to grades between 2 to 3, but pH values that varied between 6.5 and 8.0 did not affect ferning formation. The established mouse TF test protocol also displayed reproducibility and storage stability.

**Conclusions:** A TF test protocol for the mouse model was established that could be used for comparative analyses under various ocular surface disease conditions.

**Translational Relevance:** This mouse TF test protocol will facilitate the application of basic research into the mouse model to clinical care.

## Introduction

A healthy and stable tear film plays a vital role in maintaining the health of the ocular surface.<sup>1</sup> Tear fluid is a complex mixture of proteins, lipids, metabolites, and electrolytes.<sup>2,3</sup> Any change in these components can impair tear fluid functions and may be considered to be an important indication of ocular surface disease.<sup>4</sup>

Several clinical tests are available to examine tear film quantity (i.e., tear volume) and quality (i.e., tear film stability).<sup>5,6</sup> Tear quantity can be determined using Schirmer's test or the phenol red thread test,<sup>7</sup> and tear quality can be assessed by measuring tear break-up time (TBUT).<sup>8</sup> Also, tear osmolarity can reflect some aspects of the tear chemical properties and may be assessed by the TearLab Osmolarity System (TearLab Corp, Escondido, CA)<sup>9</sup> or the I-PEN Tear Osmolarity System (I-MED Pharma Inc., Dollard-des-Ormeaux,

QC, Canada). Ideally, the tests should be simple to perform, repeatable, sensitive, specific to diseases, and cost effective. However, tear fluid assessment in animal models is generally regarded as challenging due to the characteristic small volume and dynamic nature of the tears.<sup>10,11</sup> Additional drawbacks of tear analysis include low specificity and low sensitivity, as well as high cost.<sup>12</sup> For these reasons, efforts have been directed toward developing alternative methods for tear analysis, at least to serve as complementary procedures.

The tear ferning (TF) test has recently been described as a simple, fast, and inexpensive method to indirectly assess tear quality.<sup>13,14</sup> The TF test involves dropping a tear sample onto a glass slide and allowing the sample to desiccate under normal room temperature and humidity to produce a crystallization pattern that can be examined under a light microscope.<sup>15</sup> It has been reported that the test supports the clinical diagnosis of ocular surface diseases, such as Sjögren's syndrome<sup>16,17</sup> and keratoconjunctivitis.<sup>18,19</sup>

Only a few TF studies of animal models of ocular surface diseases have been reported. Silva et al.<sup>20</sup> investigated the tear ferning of healthy horses, and other researchers have applied TF tests to dogs,<sup>21</sup> capuchin monkeys,<sup>22</sup> and camels.<sup>23</sup> TF tests have not been described in mice, to the best of our knowledge, despite that mouse model being widely used for studies in ocular surface diseases. Hence, this study aimed to investigate the determining factors in tear ferning formation in healthy mice and to optimize testing conditions to establish a standardized protocol for further analyses under various ocular surface disease conditions.

## Materials and Methods

### Animals

A total of 24 healthy 8-week-old female Institute of Cancer Research (ICR) mice (purchased from Lunge Industry Co., Ltd., Taiwan) were used in this study. The mice were examined and confirmed to be without clinical or ophthalmic diseases. All mice were housed in an animal facility maintained at 20°C to 24°C with 50% to 55% humidity under a 12-hour light/12-hour dark cycle; they were given a commercial diet and water ad libitum. All animal care and treatment protocols were performed in accordance with the standard laboratory animal protocols approved by the Institutional Animal Care and Use Committee at Chung Shan Medical University, and all experimental procedures were conducted in accordance with the ARVO State-

ment for the Use of Animals in Ophthalmic and Vision Research.

### Preparation of Wash Solutions at Various Osmolarities and pH Values

The wash solutions for TF test were adjusted for various osmolarities (280, 290, 310, 330, 350, and 360 mOsm/L) and pH values (6.5, 7.0, 7.5, and 8.0) to determine their effects on ferning formation. Different amounts of NaCl were added to sterilized water to produce the wash solutions of various osmolarities (detailed formulas are shown in Supplementary Table S1). Wash solutions with different pH values were adjusted with Na<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> in sterilized water (Supplementary Table S2). All of the prepared wash solutions with specific pH values were measured for osmolarity to ensure that they were within normal range (285 ± 5 mOsm/L).

### Tear Sample Collection and Tear Ferning Formation

The mice were anesthetized with 2.5% Avertin (Sigma-Aldrich, St. Louis, MO) at 400 mg/kg by intraperitoneal injection.<sup>24</sup> Then, an aliquot of wash solution (volume detailed below) was dropped onto the ocular surface with a micropipette and washed five times by repetitive pipetting. Care was taken to avoid damage to the ocular surface and loss of wash solution. The wash solution was then recovered from the medial canthus of each mouse eye. All tear samples underwent the TF test immediately after collection without refrigeration. Each sample was placed on a horizontally positioned glass slide and allowed to dry spontaneously in an oven monitored with a digital thermo-hygrometer (LE-509RH; Yih Der, Taiwan) under controlled conditions of temperature (24°C ± 2°C) and relative humidity (rH; 46% ± 2% or 53% ± 2%) until complete dehydration. Each tear sample was collected from one eye with one wash solution. Repetitive tear sample collections were allowed from each eye after a washout period of at least 2 days.

### Relative Humidity Test

An aliquot of 2, 5, 10, or 20 μL of 0.9% NaCl saline was dropped onto the ocular surface and recovered after the washes. All tear samples were allowed to dry as previously described to determine the suitable relative humidity (46% ± 2% or 53% ± 2%). For each aliquot volume tested, six tear samples were collected from both eyes of three mice.

### Wash Solution and Volume Test

In previous literature, various wash volumes and solutions (either 0.9% NaCl saline or sterilized water) were used for TF tests in different animal models. To the best of our knowledge, there have been no published reports on mouse TF tests, so we sought to examine the effects of various wash solutions and volumes to optimize test procedures.

Different volumes (2, 5, 10, or 20  $\mu\text{L}$ ) of 0.9% NaCl saline or sterilized water were used as wash solutions to collect tear samples from both eyes of eight mice. After the washes, each collected wash solution was defined as one tear sample. Considering that some amount might be lost during the washing procedures (e.g., absorbed by the eye), the final volumes for the TF test were designated as 1, 1.5, 4.5, 9.5, or 19.5  $\mu\text{L}$ . All tear samples for the effects of wash solution and wash volume were tested at  $24^\circ\text{C} \pm 2^\circ\text{C}$  and rH of  $46\% \pm 2\%$ .

### Influence of Centrifugation

In a clinical setting, the collected tear samples are sometimes centrifuged to remove impurities such as cosmetics or dust before the TF tests are processed,<sup>25</sup> to eliminate potential interfering factors that could impede or enhance tear ferning formation. However, centrifugation is not a standard procedure in the TF test protocol, and its effects on ferning formation are largely unknown. Thus, this study also examined the effects of centrifugation on ferning formation. A 2- or 5- $\mu\text{L}$  aliquot of 0.9% NaCl saline was used as the wash solution to collect tear samples. The tear samples, centrifuged or not, were collected from both eyes of four mice. The centrifugation was set at 4000 rpm for 10 minutes at  $24^\circ\text{C} \pm 2^\circ\text{C}$ . Parallel preparation was conducted for the samples without centrifugation. All TF tests for the influence of centrifugation were conducted at  $24^\circ\text{C} \pm 2^\circ\text{C}$  and rH of  $46\% \pm 2\%$ .

### Reproducibility

The reproducibility of ferning formation produced by tear samples from the same mouse eye requires confirmation before the TF protocol can be used as a study tool. Tear samples were collected from a total of 19 eyes at three different times with a washout period of 2 days each time. A 2- $\mu\text{L}$  0.9% NaCl saline solution was used to elute each tear sample, followed by TF tests at  $24^\circ\text{C} \pm 2^\circ\text{C}$  and rH of  $46\% \pm 2\%$ .

### Storage Stability After Ferning Formation

For field study outside the laboratory, long-term storage of samples after TF testing is necessary. Unlike standard histological tissue sections that can be mounted with coverslips after staining, the same procedure would ruin tear desiccates; therefore, the stabil-

ity of tear desiccates must be determined when long-term preservation is required. A total of 12 tear desiccates that had been prepared with 2  $\mu\text{L}$  0.9% NaCl saline as the wash solution were stored at room temperature ( $22^\circ\text{C} \pm 2^\circ\text{C}$ ) and at rH of  $50\% \pm 5\%$  for 3 and 10 days, followed by an examination of their preserved condition.

### Influence of Tear Osmolarity and pH Value

The effects of different tear osmolarity and pH values on TF tests were assessed. Wash solutions (volume fixed at 2  $\mu\text{L}$ ) of different osmolarities (280, 290, 310, 330, 350, or 360 mOsm/L) and different pH values (6.5, 7.0, 7.5, or 8.0) were used to collect tear samples, followed by TF tests at  $24^\circ\text{C} \pm 2^\circ\text{C}$  and rH of  $46\% \pm 2\%$ . For each osmolarity or pH value, tear samples were collected from both eyes of five mice.

### Definition of Zones After Tear Sample Desiccation

Zones of the crystalloid formation were defined according to previously published criteria by López Solís et al.<sup>26</sup> and Traipe-Castro et al.,<sup>27</sup> where zone I is the outermost component of the desiccate. Zone II consists of a band of clear-cut crystalloid structures that appear to emerge centripetally from regularly spaced points in proximity to zone I. Zone III corresponds to the center of the desiccated tear sample and is characterized by the occurrence of typical fern-like structures. A successful TF formation was defined as complete formation of all three zones.

### Tear Ferning Image Capture and Grading

The results of tear ferning were photographed at  $40\times$  and  $100\times$  magnification under a light microscope (DM500; Leica, Wetzlar, Germany). The images were graded according to the Masmali grading scale (grades 0, 1, 2, 3, and 4).<sup>10</sup> Briefly, grade 0 contained full crystallization without spaces or gaps between the ferns; grade 1 showed a lower density of ferns and branches, with small spaces and gaps; grade 2 exhibited a greater reduction of ferns and branches, with the presence of clear spaces and gaps; grade 3 showed increased spaces and gaps and the presence of large crystals but lacked the formation of ferns; grade 4 demonstrated a loss of crystallization. A TF grade less than 2 was considered normal and over 2 was considered unhealthy.

**Table 1.** Success Rates of Tear Ferning Formation for Healthy Mice Under Different Wash Solution and Drying Volumes

Drying Volume (μL)	Wash Solution Volume (μL)			
	2	5	10	20
1	20/38 (53%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
1.5	29/57 (51%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
4.5	—	0/15 (0%)	0/15 (0%)	0/15 (0%)
9.5	—	—	0/15 (0%)	0/15 (0%)
19.5	—	—	—	0/15 (0%)

### Statistical Analysis

Statistical analysis was performed with SPSS Statistics 18.0 (IBM, Armonk, NY). One-way analysis of variance was used for comparison between groups. *P* < 0.05 was regarded as statistically significant.

## Results

### Tear Ferning Formation Under the Influence of Different Relative Humidities and Drying Volumes

After dehydration, the tear desiccates produced from 2, 5, 10, or 20 μL of 0.9% NaCl saline wash solution were examined under a 100× magnification light microscope. Two rH conditions (46% ± 2% or 53% ± 2%) were tested to optimize the protocol. At 53% ± 2% rH, a circular area of leaf-shaped crystalloid structures that surrounded the entire area of the tear desiccates readily formed in all wash volumes tested. However, in the center region encompassing zones II and III, no ferning patterns were observed (Fig. 1A). At 46% ± 2% rH, all wash volumes, except the 20-μL wash solution, produced a hyaline amorphous structure that surrounded the outermost circular area (zone I), but only the 2-μL wash solution led to formation of fern-like or branched structures in zones II and III (Fig. 1B). The results indicate that fern-like or branched structure formation in zones II and III appeared only at 46% ± 2% rH, and only the 2-μL wash volume led to complete fern formation when the entire wash solution was dried. Based on these observations, we then fixed the rH at 46% ± 2% for further analyses.

### Effects of Wash Volume on Tear Ferning Formation

To further understand whether TF formation was affected by the drying volume or wash volume, differ-

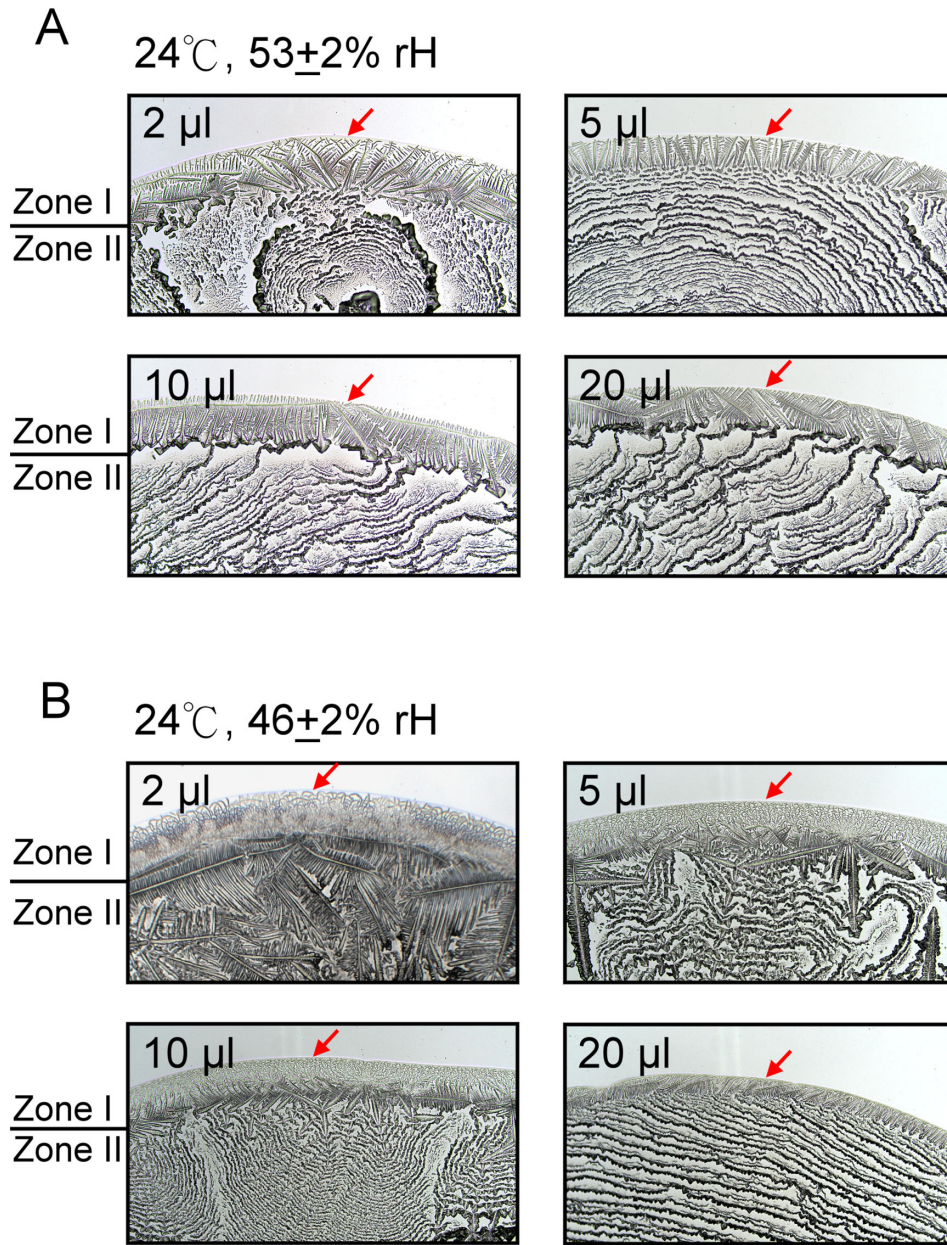
ent drying volumes from different wash volumes were examined. The results showed that wash volumes ≥ 5 μL of 0.9% NaCl saline did not produce any ferning formation under any drying conditions (Fig. 2). With the wash volume at 2 μL, both 1- and 1.5-μL drying droplets showed a complete ferning pattern containing all three parts, zones I, II, and III (Fig. 2). A systematic comparison of tear desiccates from both eyes of 24 healthy mice allowed us to identify the success rate of tear ferning forming (Table 1). Only tear desiccates produced from the 2-μL wash volume led to successful ferning formation; drying volumes of 1 μL or 1.5 μL showed success rates of 53% and 51%, respectively.

### No Tear Ferning Formation Using Sterilized Water as Wash Solution

When using sterilized water as the wash solution, there was no ferning formation at volumes of 2, 5, 10, or 20 μL (Fig. 3). The tear desiccates showed only irregular shapes, with the formation of crystals mainly concentrated on the edges of the droplets.

### Influence of Tear Sample Centrifugation on Ferning Formation

The effect of centrifugation on TF formation was investigated by comparing results for procedures with or without centrifugation. After centrifugation at 4000 rpm for 10 minutes, the supernatants of tear samples were dropped onto glass slides to desiccate. At a wash volume of 5 μL, centrifugation did not affect the results, as there was no ferning formation from either the 1-μL or 1.5-μL drying volumes (Fig. 4). However, at a wash volume of 2 μL, desiccates prepared from the 1-μL and 1.5-μL drying volumes showed worse patterns due to centrifugation. Most of the crystal formation was concentrated around the edge of the tear droplet, and ferning structures did not appear at zones II or III (Fig. 4).



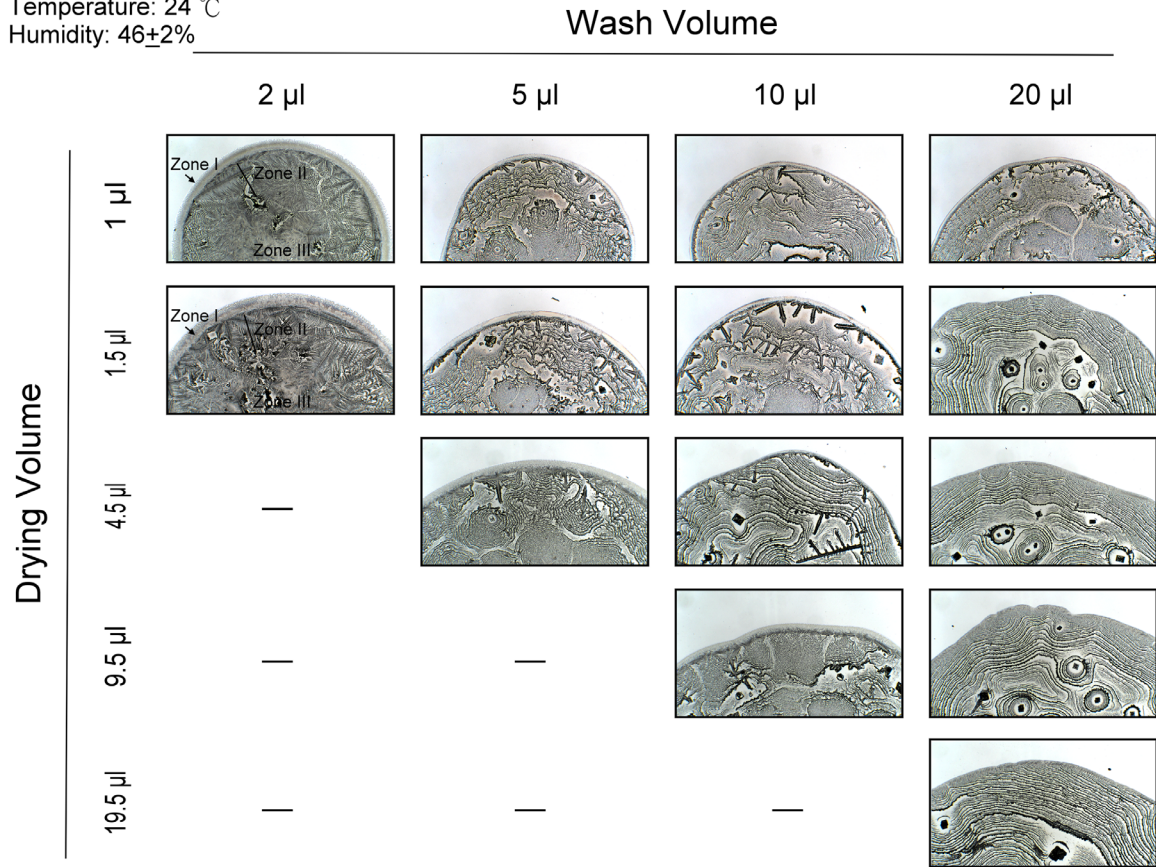
**Figure 1.** Representative morphological patterns formed in tear ferning under different settings. Tear desiccates produced by 2 to 20 µL of collected samples were formed on glass slides after drying in an oven under controlled conditions. Digital images of the desiccates were photographed under 100× magnification light microscopy. **(A)** Ferning patterns at 24°C and at 53% ± 2% rH show a leaf-shaped pattern in the outmost region, whereas the central region shows only wavy patterns. **(B)** At 24°C and at 46% ± 2% rH, desiccates from the 2-µL drying volume showed typical ferning patterns containing both zones I and II, whereas those from the other drying volumes displayed only irregular patterns.

### Reproducibility of TF Test in Mice

When the tear ferning conditions had been optimized with regard to relative humidity, wash volume, dry volume, and wash solution, we observed that the morphological characteristics of the tear desiccates were generally very similar if ferning forma-

tion was successful. We then investigated whether the successful ferning formations produced by sample aliquots taken from the same mouse eye were reproducible. Tear samples were collected from a total of 19 eyes at three different times with a washout period for 2 days and were subjected to TF tests. Successful ferning formation for each of the three times was seen

Temperature: 24 °C  
Humidity: 46±2%



**Figure 2.** The effects of wash volume on tear ferning formation. Only 2 µL of 0.9% NaCl saline wash volume led to successful tear ferning formation containing zones I, II, and III (40× magnification). The 1-µL and 1.5-µL drying volumes produced similar ferning formations and success rates (53% and 51%, respectively).

in five of the 19 eyes (26%); it was seen in five eyes (26%) for two of the times, and in four eyes (21%) for one time. Five eyes did not achieve any successful ferning formation (26%) (Fig. 5).

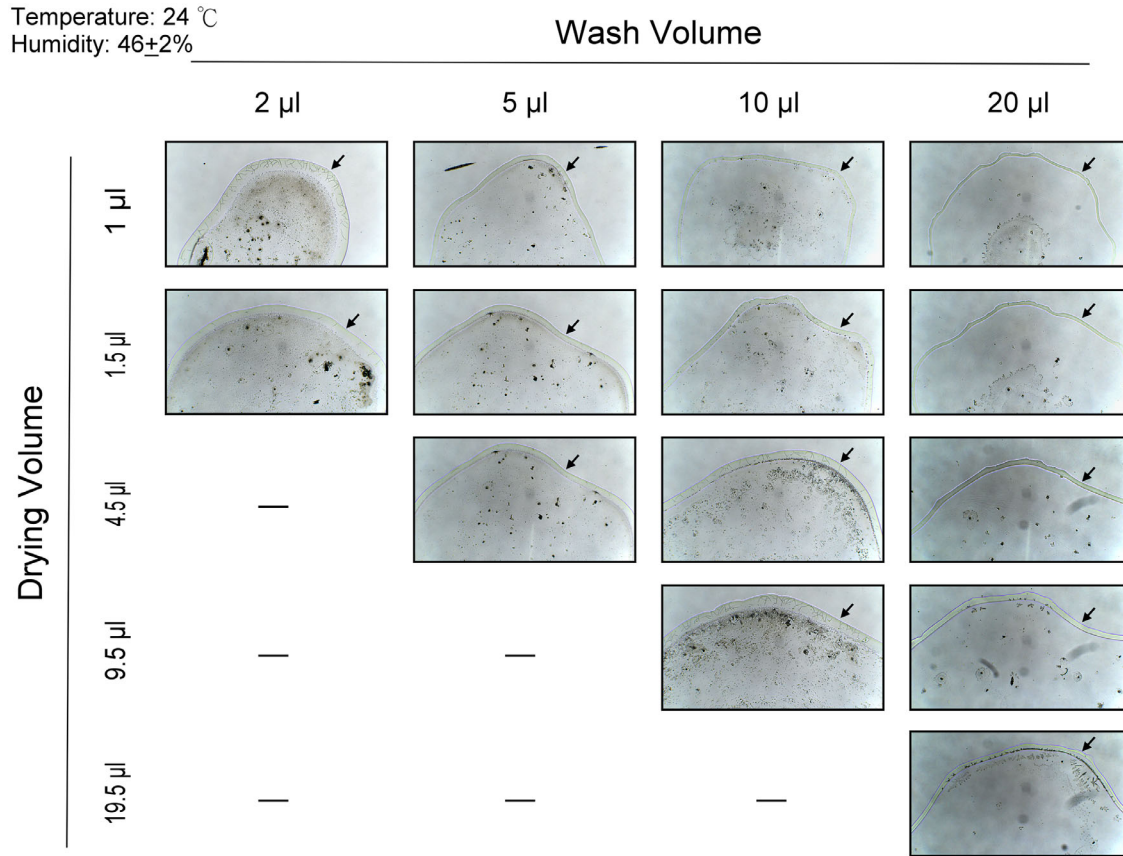
### Storage Stability of Tear Desiccates from Healthy Mouse Eyes

Unlike stained specimens of histological sections, tear ferning preparations cannot be mounted with coverslips for long-term storage; therefore, the stability of tear desiccates must be determined, particularly at normal room temperature (22°C ± 2°C) and rH (50% ± 5%). We examined 12 tear ferning preparations to evaluate the stability of tear desiccates. On the first and third day after drying, there were no changes in the ferning patterns. On the tenth day, five of 12 tear desiccates exhibited slightly changed morphology and color (Fig. 6, red arrow). The results indicate that tear desiccates can maintain their ferning patterns for 10 days.

### Influence of Tear Osmolarity and pH Value on Ferning Formation

The wash solutions were prepared with a variety of osmolarity and pH values to determine potential effects on ferning formation. The results showed statistically significant differences in the Masmali grading scale when the scale at 360 mOsm/L was compared with that at 280 mOsm/L or at 290 mOsm/L (Fig. 7). Furthermore, as detailed in Table 2, more grading scales shifted from grades 0 and 1 to grades 2 and 3 above 330 mOsm/L. Under the influence of 310 mOsm/L, 50% of tear samples were graded 0 to 1 and another 50% were graded 2 to 3. At 330 mOsm/L, 85.7% reached grades 2 and 3, leaving only 14.3% as grades 0 and 1. Although a deviation of this trend was observed with osmolarity at 350 mOsm/L, 85.7% of the samples were graded as 2 or 3 under the influence of 360 mOsm/L.

The influence of pH values on ferning formation, however, was not as obvious as that of osmolarity and showed no statistically significant difference among all



**Figure 3.** Tear desiccates prepared by using sterilized water as wash solution. No successful tear ferning formation was observed when sterilized water was used as the wash solution. Crystalloid formations were gathered near the edges of the desiccates, indicated by *black arrows* (40× magnification).

**Table 2.** Distribution of Masmali Scale Grades for Tear Ferning Under Different Osmolarities in Healthy Mice

Masmali Scale, <i>n</i> (%)	Osmolarity (mOsm/L)					
	280	290	310	330	350	360
Grade 0	5 (71.4)	3 (33.3)	2 (33.3)	1 (14.3)	0 (0)	0 (0)
Grade 1	1 (14.3)	5 (55.6)	1 (16.7)	0 (0)	3 (50)	1 (14.3)
Grade 2	1 (14.3)	1 (11.1)	2 (33.3)	4 (57.1)	2 (33.3)	1 (14.3)
Grade 3	0 (0)	0 (0)	1 (16.7)	2 (28.6)	0 (0)	5 (71.4)
Grade 4	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	0 (0)
Total	7 (100)	9 (100)	6 (100)	7 (100)	6 (100)	7 (100)

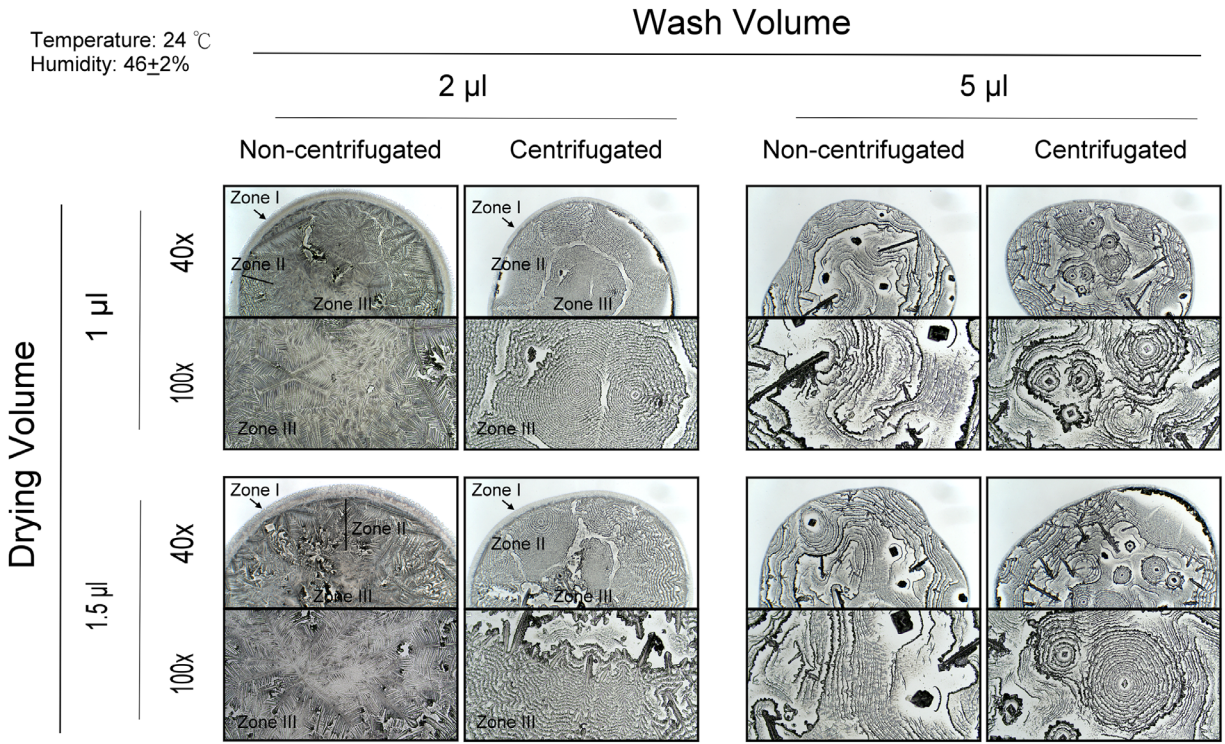
pH values (Fig. 8). There was no obvious shift of grades from 0 or 1 to 2 or 3 as pH values were elevated from a range of 6.5 to 7.0 to a range of 7.5 to 8.0 (Table 3).

## Discussion

The TF test has been reported to be a simple tool to assess tear quality for evaluating dry eye conditions and

predicting contact lens tolerance.<sup>13</sup> Previous reports have described the application of TF tests in dogs,<sup>21,28</sup> horses,<sup>20</sup> and camels.<sup>23</sup> To the best of our knowledge, the use of TF tests in mice is being reported here for the first time.

Collecting tears can be difficult, especially in mice, due to the very little amount of tear available; it is even more challenging under pathological lower tear production conditions. In previous studies, applying



**Figure 4.** Influence of centrifugation on ferning formation. The desiccates were produced from tear sample aliquots with 2- or 5- $\mu$ L wash volume and prepared with or without centrifugation at 4000 rpm for 10 minutes. All images in this representative series are presented at the same magnifications (40 $\times$  and 100 $\times$ ). All tear desiccates taken from the centrifuged samples showed deteriorated ferning patterns compared with samples that were not centrifuged.

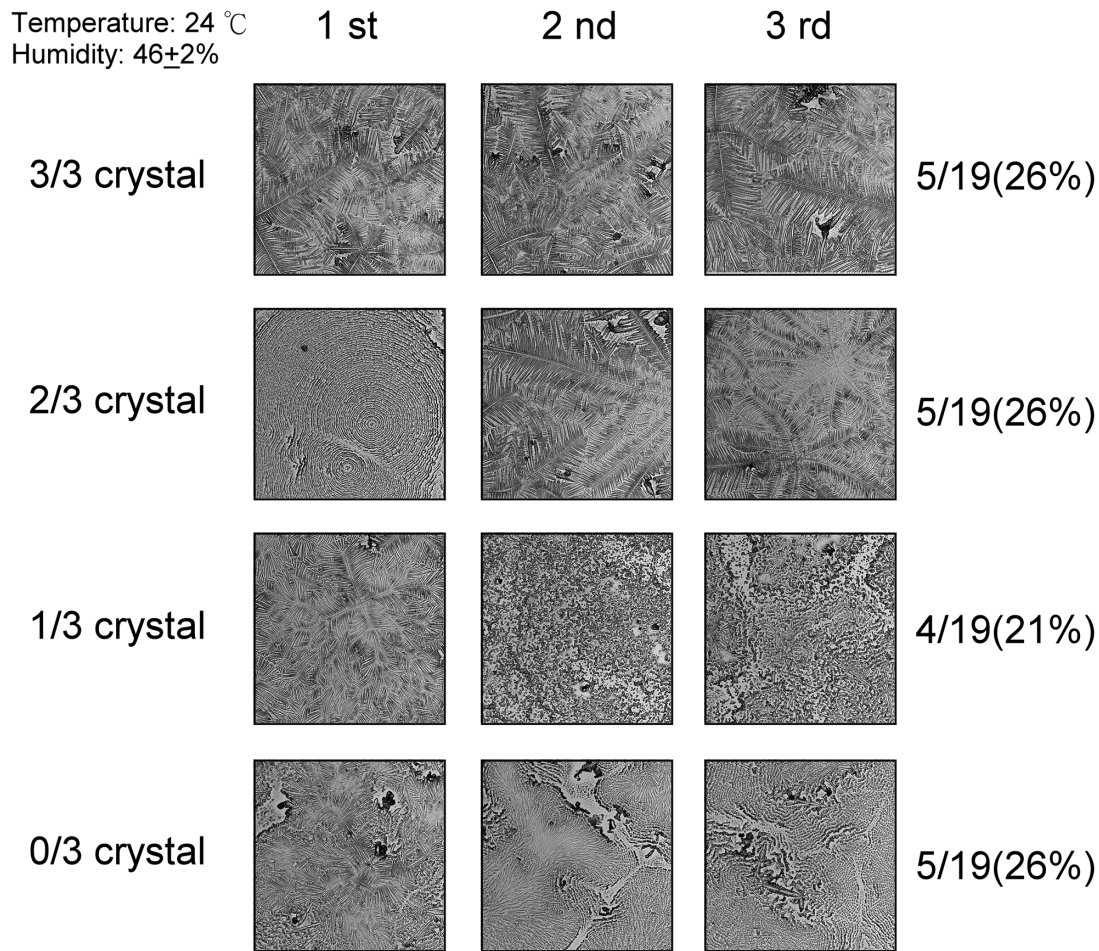
**Table 3.** Distribution of Masmali Scale Grades for Tear Ferning Under Different pH Values in Healthy Mice

Masmali Scale, <i>n</i> (%)	pH			
	6.5	7.0	7.5	8.0
Grade 0	2 (28.6)	3 (50)	4 (66.7)	5 (83.3)
Grade 1	4 (57.1)	2 (33.3)	2 (33.3)	1 (16.7)
Grade 2	1 (14.3)	1 (16.7)	0 (0)	0 (0)
Grade 3	0 (0)	0 (0)	0 (0)	0 (0)
Grade 4	0 (0)	0 (0)	0 (0)	0 (0)
Total	7 (100)	6 (100)	6 (100)	6 (100)

a capillary tube to the nasal conjunctival fornix of the lower eyelid has been a common method for tear collection.<sup>9</sup> Another method utilizing a micropipette was suggested by Norm<sup>29</sup> and was applied to the research by Oriá et al.<sup>21</sup> in dogs. In the present study, we collected mouse tears with a micropipette using an additional 0.9% NaCl saline solution to wash the ocular surface and recover the liquid. Although the use of additional wash solution could affect tear ferning formation, our results indicated that this approach was still workable after optimization.

Tear ferning formation is influenced by a number of exogenous and endogenous factors. Evaporation of tear droplet water content results in a gradual increase of tear component concentration until conditions allow for ferning formation. Environmental factors such as air flow, temperature, and relative humidity could affect the evaporation rate in an open space.<sup>30</sup> To reduce such an influence, it has been suggested that TF tests be performed in an oven with set conditions.<sup>31,32</sup> Nevertheless, how the oven temperature and relative humidity should be optimized for tear ferning has remained unclear. It has been suggested that humidity higher than 50% and temperatures above 26°C would result in deterioration of the ferning patterns.<sup>31</sup> Traipe-Castro et al.<sup>27</sup> reported that under lower air pressure or at a higher temperature (30°C) the time of desiccation was reduced by half, but the resulting tear desiccates lacked distinctive zones II and III. In this study, taking into consideration various environmental factors, we showed that a single tear sample of normal mouse can display different ferning outcomes under different drying conditions. At 53%  $\pm$  2% rH, only a leaf-shaped crystalloid structure appeared at the outmost layer zone I, with no appearance in zones II or III. In contrast, at 46%  $\pm$  2% rH, tear desiccates





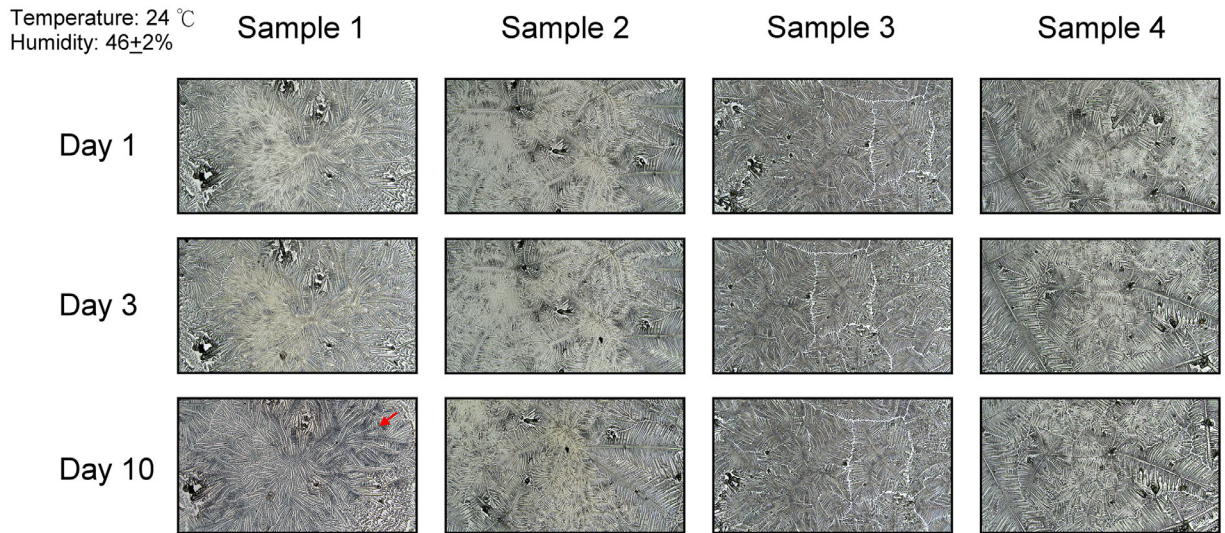
**Figure 5.** Tear samples from healthy mouse exhibited different success rates of ferning formation. Representative images of TF test results from three tear samples taken from a single eye. After the first tear sample collection, the second and the third time samples were collected, each after a 2-day washout period. The images show that ferning formation ranged from being successful all three times to being not at all successful; the numbers and percentages indicated on the right are for the 19 samples tested. Note that all of the successful ferning patterns were similar. Digital images of desiccates were captured at 100× magnification.

produced from 2 μL of tear sample showed typical ferning patterns, similar to those described in other species. Thus, the TF test protocol for the mouse model can be optimized accordingly.

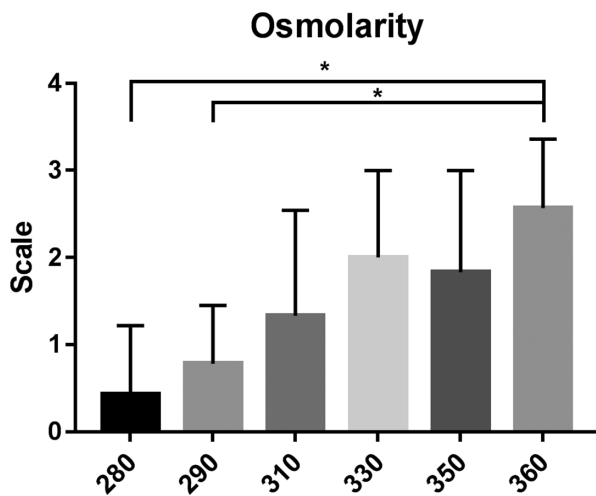
Apart from environmental factors, TF tests may also be affected by the aggregation of contents in tear samples. The tears collected from human eyes were found to contain cellular debris, cosmetic products, and often lumps of insoluble mucus. These materials may aggregate in the center of the tear desiccates and disturb the ferning patterns.<sup>20</sup> In mice tear samples, dust particles from the environment are also likely to alter the crystallization pattern, which may be avoided by eliminating the particles after centrifugation.<sup>25</sup> Nevertheless, our results indicate that centrifugation reduced the success rate of ferning formation. It is speculated that centrifugation could cause the precip-

itation of tear components, leading to ferning interference.

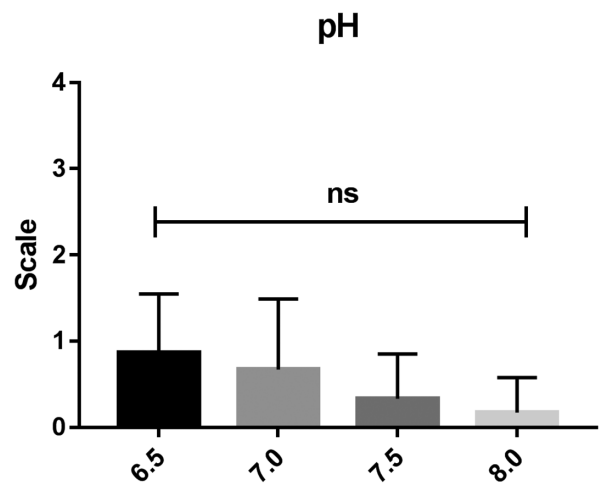
Electrolytes and their interactions with macromolecules in tear samples may also affect tear ferning patterns.<sup>20,32</sup> It has been proposed that ion concentrations, especially the ratio of monovalent Na<sup>+</sup> and K<sup>+</sup> to divalent Ca<sup>++</sup> and Mg<sup>++</sup>, play a key role in tear ferning formation.<sup>25,33</sup> Protein or mucin components may also affect tear ferning by lowering the surface tension of the drop<sup>34</sup> or limiting the periphery of the tear sample.<sup>35</sup> All of the aforementioned tear characteristics contribute to osmolarity, which in turn affects tear ferning formation. Interestingly, our results in the present study indicate that osmolarity over 330 mOsm/L promoted a shift of ferning grades from grades 0 and 1 to grades 2 and 3. Specifically, the ferning scale at 360 mOsm/L showed



**Figure 6.** Stability of dried tear desiccates at different storage times. The tear desiccates were kept at normal room temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) and rH ( $50\% \pm 5\%$ ). At day 10, slightly changed ferning patterns and colors were observed (red arrow) in five out of 12 preparations. Digital images of the desiccates were captured at  $100\times$  magnification.



**Figure 7.** Influence of osmolarity on ferning formation. The Masmali grading scale results were significantly increased with elevated osmolarity. The detailed distribution of grading scales is shown in Table 2. Values are shown as mean  $\pm$  SD. \* $P < 0.05$  versus osmolarity at 360 mOsm/L.



**Figure 8.** Influence of pH values on ferning formation. The results showed generally similar grading scales between pH 6.5 to 8.0. The detailed distribution of grading scales is listed in Table 3. Values are shown as mean  $\pm$  SD; ns, non-significant.

statistically significant differences when compared with those at 280 mOsm/L and 290 mOsm/L. This finding is coincident with osmolarity higher than 330 mOsm/L being defined as hyperosmolarity in previous literature<sup>36</sup> and suggests the applicability of TF tests as a rough osmolarity indicator for dry eye studies, at least in the mouse model. On the other hand, our results showed no relationship between pH values (range, 6.5–8.0) and ferning formation, thus negating the potential use of TF tests to indicate tear pH values.

To extend the use of TF tests as indicators of tear quality, the reproducibility of ferning patterns and success rates should be determined. In a previous report, López Solís et al.<sup>26</sup> showed that multiple desiccates produced from a single sample display similarities in their morphological features. Another report by Masmali et al.<sup>37</sup> found no significant differences in the ferning patterns of tear samples collected at different times of the day. The results from this study showed consistent patterns when ferning formation

was successful, in agreement with previous reports. However, in terms of success rate, only five of the 19 tear samples (26%) displayed successful ferning formation for all three TF tests. Five of the 19 tear samples (26%) did not show any ferning formation in any of the three TF tests. These results may reflect the biological characteristics of normal mouse tear samples. Alternatively, the 2-day washout period in the protocol may not be sufficient and thus affected consecutive tear ferning formations. If the normal mouse tear characteristics vary significantly, use of the TF test as a tool for dry eye evaluation could be considerably limited. It is likely that only 26% of the mice displaying stable tear ferning could be included in experiments as normal controls.

Due to the low tear volume in mice, we tried to collect tear samples by using extra wash solution such as 0.9% NaCl saline or sterile water to elute the tear components on the ocular surface. When the volume of wash solution exceeded 2  $\mu$ L (whether 0.9% NaCl saline or sterilized water), none of the dried tear desiccates formed any fern-like pattern. We speculate that excessive wash solution may have diluted the components of the tear samples and increased the drying time, which is disadvantageous for crystalloid formation. Likewise, excessive dilution of tear components led to total failure of ferning formation when sterilized water was used as the wash solution.

The validity and interpretation of TF test results must be determined before TF tests can be used as a complementary tool for ocular surface health diagnosis, in either clinical settings or experimental animal models. The success rate of crystalloid formation, in humans as well as in animals, may also be affected by many other factors, including gender or age, which were not addressed in the present study. Another limitation of the present study is that the tear samples were derived from only healthy mouse eyes and were not compared with those from other ocular surface conditions, such as, for example, dry eye and allergic conjunctivitis. Thus, further studies are warranted to better understand ferning patterns in mice with different ophthalmic abnormalities and to correlate TF tests with other clinical tests such as TBUT and Schirmer's test.

## Conclusions

The results from this study show that tear ferning occurs with mouse tear samples, and a mouse TF test

protocol was established. Despite some limitations, the TF test could be a valuable tool for clinical and research use in small animals with little tear volume.

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