Sample preparation for mass spectrometric analysis

Lyophilization - For optimal comparability and facilitated further processing, it was necessary to equalize each sample to 15 µg protein per reaction tube by lyophilization. The required sample volume was calculated from the protein concentration determined by amino acid analysis, transferred to a reaction tube, and frozen at -80 °C. This frozen sample was transferred to a metal stand precooled to -80 °C and placed in the lyophilization chamber (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). After creating a vacuum, the unit was turned on and drying started. Samples were dried overnight and stored at -80 °C until further usage.

Gel electrophoresis - Each lyophilized sample was resuspended with 2.5 µL 10x Bolt™ Sample Reducing Agent (Thermo Fisher Scientific Inc.), 6.25 µL 4x LDS sample buffer (AppliChem GmbH), and 16.25 µL distilled water. The sample mixture was shaken to obtain a homogeneous solution. Subsequently, the samples were incubated at 95 °C and 350 rpm for 5 min in a thermomixer (Eppendorf AG). Before loading onto the gel, each sample was centrifuged to recover condensed liquid [1]. For gel electrophoresis, NuPage™ 10 % Bis-Tris gels (Thermo Fisher Scientific Inc.) were used. Gel electrophoresis was performed in step 1 at 200 mA, 200 W, and 50 V for 20 min. In step 2, the voltage was increased to 120 V (2 min). 1x MES buffer (Thermo Fisher Scientific Inc.) was used as running buffer.

Protein staining - The gel was stained with Coomassie brilliant blue (SimpleBlue™ SafeStain, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions.

Destaining - Gel bands were separated from each other on a glass plate using a scalpel and transferred to glass vials. For destaining and to adjust the samples to the correct pH, gel bands were washed according to Steinbach *et al*. [2]. Finally, gel pieces were dried in a vacuum concentrator (Concentrator plus, Eppendorf AG).

In-gel digestion and peptide extraction - For tryptic digestion, 6 µL (0.033 µg/µL) trypsin dissolved in ammonium bicarbonate was added to the gel pieces. Then, 14 µL of ammonium bicarbonate was applied, ensuring accessibility for the trypsin. Samples were incubated for 15 h at 37 °C in a thermomixer (Eppendorf AG). Digestion was stopped by adding an extraction solution (40 µL 50 % (v/v) acetonitrile and 50 % (v/v) 0.1 % trifluoroacetic acid (TFA). For peptide extraction, the samples were treated in an ice-cooled ultrasonic bath for 15 min. Subsequently, the solution was removed and transferred to a new glass vial. This procedure was repeated with 40 µL of extraction solution. Afterwards, samples were dried in the vacuum concentrator. Finally, samples were resuspended with 20 µL 0.1 % TFA and peptide

concentration determined by amino acid analysis. For mass spectrometry analysis 400 ng per sample were used. Each sample was made up to 15 µL with 0.1 % TFA and 1 µL of indexed retention time peptides (iRT) (Biognosis AG, Switzerland) was added.

High performance liquid chromatography and mass spectrometry

High performance liquid chromatography (HPLC) - HPLC was performed on the UltiMate™ 300 RSLC nano system (Thermo Fisher Scientific Inc.). For this, 400 ng peptide was injected using a sampler (Dionex Softron GmbH, Germany) at 60 °C and a flow rate of 30 µL/min. After concentration of the peptides for 7 min on a reversed phase precolumn (Acclaim PepMap, C18 phase, 100 µm x 2 cm, particle size 5 µm, pore size 100 Å), peptides were transferred to a separation column (Acclaim PepMap, 75 µm x 25 cm, particle size 2 µm, pore size 100 Å). Peptides were separated by a gradient pump using buffer A (0.1 % (v/v) formic acid) and buffer B (0.1 % (v/v) formic acid, 84 % (v/v) acetonitrile). The gradient ranged from 5 %-95 % buffer B. A flow rate of 0.4 µL/min was applied for 150 min. The column was then equilibrated with 5 % buffer B. After each sample, a 50 min wash step was performed, which was designed to remove residues from the column.

Mass spectrometric analysis - Peptides eluted from HPLC were transferred online by an electrospray ion source to the Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific Inc.) used. Ionization was performed at 1.5 kV and 275 °C. To obtain consistently comparable results, an internal standard was measured at regular intervals between samples.

DDA measurement - To complement the spectral library of Guntermann *et al*. [1] ten replicates of a master mix consisting of 128 pooled fractionated samples were measured utilizing the data-dependent acquisition (DDA) method. For this purpose, a full spectrum was first acquired in a mass range from 350 Da to 1400 m/z at a resolution of 60,000. For the internal recalibration, an automatic gain control-target (ACG-target) was employed. In each 80 ms cycle, the ten most abundant precursors were used to record additional MS/MS spectra (top 10 method). These were isolated and fragmented using a higher collision dissociation (HCD) cell. The normalized collision energy (NCE) was 28 %. Subsequently, the fragment ions obtained were analyzed in the Orbitrap in a 1.6 m/z wide isolation window at a resolution of 30,000. The fixed first mass was set to 100 m/z. To avoid re-measuring ions that had already fragmented, dynamic exclusion was employed to create a 30 second window in which these ions were excluded.

DIA measurement - Mass spectrometric measurements were performed on the Q Exactive™ HF system in data-independent acquisition (DIA) mode to ensure high reproducibility, sensitivity, and accuracy of quantification. The full spectrum scan ranged from 350 – 1200 m/z and was divided into 34 m/z wide regions, each had an overlap of 1 m/z. The full spectrum was acquired at a resolution of 120,000, an ACG target of 3e6, and an automatic injection time. Fragment ion formation was by HCD, with an NCE of 30 %, 27 %, and 25.5 % and a resolution of 30,000. The standard charge state was set to ≥ +2, while the first fixed mass was set to 200 m/z.

Use of a spectral library

The spectral library used consisted of ten technical replicates from a tear fluid pool of 18 healthy volunteers. The spectra were compared with those from a human proteome database from Uniprot/SwissProt (uniprot_complete-homo_sapiens_20180705, number of entries: 73,045 as of 08/17/2018). For the generation of this spectral library, the same settings were adopted as previously used by Guntermann *et al.* [1].

Table S1A: Tear flow distance data of oculus sinister (OS) with the intra- and inter-individual coefficient of variation. Listed are the raw data of the tear flow distances from 18 subjects for OS at four time points (8 am, 12 am, 6 pm, 10 pm) on day 1 and 2, as well as the mean values of the individual subjects of the respective days (all values are given in mm). Moreover, the intra-individual coefficient of variation (CVi) and the inter-individual coefficient of variation (CVs) on both days are shown using the coefficient of variation (in percent).

Oculus sinister														
Subject ID	**Day 1**							**Day 2**						
	8 am (mm)	**12 am (mm)**	**6 pm (mm)**	**10 pm (mm)**	**Mean value (mm)**	**Cvi (%)**	**CVs (%)**	**8 am (mm)**	**12 am (mm)**	**6 pm (mm)**	**10 pm (mm)**	**Mean value (mm)**	**Cvi (%)**	**CVs (%)**
1	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
2	28	30	30	30	29.50	2.94		15	22	20	12	17.25	22.96	
3	22	25	25	17	22.25	14.69		5	3	0	12	5.00	88.32	
4	30	30	30	30	30.00	0.00		11	28	19	22	20.00	30.62	
5	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
6	2	7	6	3	4.50	45.81		7	2	6	10	6.25	45.78	
7	15	5	8	6	8.50	45.94		11	0	2	1	3.50	125.36	
8	12	12	12	5	10.25	29.57		19	14	8	6	11.75	43.55	
9	30	30	30	30	30.00	0.00	11.11	30	30	30	30	30.00	0.00	30.59
10	30	30	25	30	28.75	7.53		30	30	12	30	25.50	30.57	
11	30	30	30	30	30.00	0.00		30	30	30	22	28.00	12.37	
12	30	5	30	18	20.75	49.78		2	7	30	30	17.25	74.62	
13	30	30	30	30	30.00	0.00		30	21	14	30	23.75	28.30	
14	15	14	25	30	21.00	32.12		25	15	9	10	14.75	42.98	
15	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
16	30	29	3	30	23.00	50.24		22	30	30	30	28.00	12.37	
17	30	7	1	30	17.00	77.48		30	0	1	0	7.75	165.84	
18	20	20	30	30	25.00	20.00		5	10	14	20	12.25	44.85	

Table S1B: Tear flow distance data of oculus dexter (OD) with the intra- and inter-individual coefficient of variation. Listed are the raw data of the tear flow distances from 18 subjects for OD at four time points (8 am, 12 am, 6 pm, 10 pm) on day 1 and 2, as well as the mean values of the individual subjects of the respective days (all values are given in mm). Moreover, the intra-individual coefficient of variation (CVi) and the inter-individual coefficient of variation (CVs) on both days are shown using the coefficient of variation (in percent).

Oculus dexter														
Subject ID	**Day 1**							**Day 2**						
	8 am (mm)	**12 am (mm)**	**6 pm (mm)**	**10 pm (mm)**	**Mean value (mm)**	**CVi (%)**	**CVs (%)**	**8 am (mm)**	**12 am (mm)**	**6 pm (mm)**	**10 pm (mm)**	**Mean value (mm)**	**CVi (%)**	**CVs (%)**
1	30	30	30	30	30.00	0.00		25	30	30	25	27.50	9.09	
2	30	15	14	20	19.75	32.10		5	7	10	10	8.00	26.52	
3	20	15	30	30	23.75	27.35		3	12	2	10	6.75	64.04	
4	30	29	30	29	29.50	1.69		11	22	14	30	19.25	38.42	
5	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
6	2	3	2	1	2.00	35.36		4	0	2	3	2.25	65.73	
7	9	15	6	4	8.50	48.86		30	3	3	4	10.00	115.54	
8	30	15	27	15	21.75	31.42		16	16	12	7	12.75	29.02	
9	30	30	30	30	30.00	0.00	21.61	25	30	30	30	28.75	7.53	27.77
10	30	30	30	30	30.00	0.00		26	30	18	30	26.00	18.84	
11	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
12	30	7	30	22	22.25	42.21		3	7	30	30	17.50	71.88	
13	30	30	30	30	30.00	0.00		30	25	18	30	25.75	19.10	
14	20	15	30	27	23.00	25.54		18	17	7	11	13.25	33.91	
15	30	30	30	30	30.00	0.00		30	30	30	30	30.00	0.00	
16	30	15	22	30	24.25	25.81		20	30	30	30	27.50	15.75	
17	10	5	12	3	7.50	48.53		4	3	3	9	4.75	52.37	
18	10	15	10	13	12.00	17.68		7	8	22	25	15.50	52.11	

Table S2: Intra-time point and intra-day coefficient of variation for the tear flow rate. The measure of dispersion within each time point, the intra-time point coefficient of variation, is referred to as CVt, while the intra-day coefficient of variation is referred to as CVd (in percent).

Time point	Day 1		Day 2	
	CVt (%)	CVd (%)	CVt (%)	CVd (%)
8 am	36.18	43.38	56.47	59.29
12 am	47.16		62.11	
6 pm	43.95		63.24	
10 pm	42.80		53.06	

Table S3A: Tear fluid protein concentration data of oculus sinister (OS) with the intra- and inter-individual scatter measure. Listed are the raw protein concentration data from 18 subjects for OS at four time points (8 am, 12 am, 6 pm, 10 pm) on day 1 and 2, as well as the mean values of the individual subjects of the respective days (all values are given in µg/µL). Moreover, the intra-individual scatter measure (CVi) and the inter-individual scatter measure (CVs) on both days are shown using the coefficient of variation (in percent).

Oculus sinister														
Subject ID	**Day 1**							**Day 2**						
	8 am (µg/µL)	**12 am (µg/µL)**	**6 pm (µg/µL)**	**10 pm (µg/µL)**	**Mean value (µg/µL)**	**CVi (%)**	**CVs (%)**	**8 am (µg/µL)**	**12 am (µg/µL)**	**6 pm (µg/µL)**	**10 pm (µg/µL)**	**Mean value (µg/µL)**	**CVi (%)**	**CVs (%)**
1	0.48	0.46	0.53	0.45	0.48	6.69		0.26	0.51	0.29	0.39	0.36	30.58	
2	0.39	0.47	0.47	0.40	0.43	9.27		0.24	0.22	0.30	0.29	0.26	15.52	
3	2.17	1.17	1.67	1.25	1.57	25.41		0.69	0.92	0.36	2.00	0.99	71.67	
4	0.64	0.65	0.68	0.51	0.62	10.56		0.31	0.42	0.78	0.68	0.55	40.12	
5	0.79	0.32	0.82	0.79	0.68	30.65		0.61	1.06	0.83	0.97	0.87	22.95	
6	0.21	0.04	0.32	0.20	0.19	52.34		0.17	0.16	0.21	0.23	0.19	17.96	
7	0.14	0.14	0.21	0.20	0.17	19.45		0.13	0.09	0.32	0.11	0.16	65.95	
8	0.27	0.34	0.41	0.65	0.42	34.37		0.26	0.20	0.30	0.23	0.25	18.27	
9	0.68	0.62	0.61	0.76	0.67	8.94	19.15	0.60	0.46	0.48	0.40	0.49	17.40	25.23
10	0.30	0.41	0.24	0.33	0.32	20.02		0.27	0.33	0.16	0.26	0.25	28.56	
11	0.52	0.55	0.73	0.82	0.65	18.85		0.69	0.44	0.43	0.44	0.50	25.20	
12	0.42	0.20	0.53	0.34	0.37	31.61		0.13	0.27	0.51	0.39	0.32	50.59	
13	0.48	0.56	0.65	0.56	0.56	10.91		0.43	0.44	0.47	0.60	0.49	15.74	
14	0.65	0.59	0.63	0.91	0.69	18.31		0.58	0.57	0.34	0.34	0.46	29.78	
15	0.46	0.38	0.52	0.39	0.44	12.80		0.37	0.49	0.48	0.55	0.47	15.53	
16	0.28	0.42	0.16	0.51	0.34	39.49		0.24	0.35	0.36	0.46	0.35	25.27	
17	0.51	0.26	0.25	0.66	0.42	41.65		0.58	0.21	0.29	0.20	0.32	55.34	
18	0.30	0.27	0.28	0.29	0.28	3.48		0.28	0.28	0.45	0.34	0.34	23.37	

Table S3B: Tear fluid protein concentration data of oculus dexter (OD) with the intra- and inter-individual scatter measure. Listed are the raw protein concentration data from 18 subjects for OD at four time points (8 am, 12 am, 6 pm, 10 pm) on day 1 and 2, as well as the mean values of the individual subjects of the respective days (all values are given in µg/µL). Moreover, the intra-individual scatter measure (CVi) and the inter-individual scatter measure (CVs) on both days are shown using the coefficient of variation (in percent).

Oculus dexter														
Subject ID	**Day 1**							**Day 2**						
	8 am (µg/µL)	**12 am (µg/µL)**	**6 pm (µg/µL)**	**10 pm (µg/µL)**	**Mean value (µg/µL)**	**CVi (%)**	**CVs (%)**	**8 am (µg/µL)**	**12 am (µg/µL)**	**6 pm (µg/µL)**	**10 pm (µg/µL)**	**Mean value (µg/µL)**	**CVi (%)**	**CVs (%)**
1	0.53	0.38	0.46	0.46	0.46	11.14		0.23	0.49	0.26	0.32	0.32	36.41	
2	0.36	0.26	0.35	0.42	0.34	16.50		0.16	0.14	0.22	0.21	0.18	20.44	
3	0.77	0.53	1.08	1.52	0.97	38.08		0.24	0.52	0.18	0.70	0.41	58.91	
4	1.66	1.65	1.45	1.23	1.50	11.61		0.85	0.86	0.42	0.80	0.73	28.60	
5	1.27	1.18	0.85	1.24	1.14	14.65		0.58	0.89	0.97	1.01	0.86	22.41	
6	0.16	0.36	0.25	0.26	0.26	27.89		0.13	0.07	0.15	0.17	0.13	34.64	
7	0.16	0.27	0.19	0.16	0.19	22.95		0.42	0.13	0.21	0.25	0.25	47.69	
8	0.31	0.38	0.45	0.38	0.38	13.34		0.25	0.18	0.34	0.37	0.28	30.18	
9	0.62	0.70	0.51	0.63	0.61	11.20	15.58	0.38	0.47	0.55	0.54	0.49	16.15	24.16
10	0.31	0.27	0.28	0.25	0.28	7.36		0.17	0.27	0.18	0.26	0.22	24.71	
11	0.66	0.64	0.86	0.68	0.71	12.30		0.52	0.55	0.35	0.64	0.51	23.61	
12	0.36	0.26	0.47	0.34	0.35	21.24		0.12	0.22	0.54	0.43	0.33	58.74	
13	0.81	0.52	0.72	0.57	0.65	17.94		0.46	0.37	0.49	0.60	0.48	19.76	
14	0.42	0.60	0.90	0.63	0.64	26.87		0.37	0.48	0.38	0.37	0.40	14.03	
15	0.45	0.39	0.47	0.40	0.42	7.59		0.40	0.44	0.52	0.49	0.46	11.86	
16	0.36	0.29	0.33	0.47	0.36	18.10		0.26	0.43	0.41	0.52	0.40	26.88	
17	0.28	0.28	0.32	0.32	0.30	7.37		0.21	0.21	0.26	0.33	0.25	22.76	
18	0.22	0.54	0.41	0.41	0.40	29.06		0.18	0.32	0.30	0.30	0.27	23.55	

Table S4: Intra-time point and intra-day coefficient of variation for the tear fluid protein concentration. The measure of dispersion within each time point, the intra-time point coefficient of variation, is referred to as CVt, while the intra-day coefficient of variation is referred to as CVd (in percent).

Time point	Day 1		Day 2	
	CVt (%)	CVd (%)	CVt (%)	CVd (%)
8 am	94.09	73.36	67.85	63.59
12 am	75.99		59.33	
6 pm	70.73		51.42	
10 pm	69.58		85.51	

References

(1) Guntermann, A.; Steinbach, S.; Serschnitzki, B.; Grotegut, P.; Reinehr, S.; Joachim, S. C.; Schargus, M.; Marcus, K.; May, C. Human tear fluid proteome dataset for usage as a spectral library and for protein modeling. *Data Brief* **2019**, *23*, 103742. DOI: 10.1016/j.dib.2019.103742.
(2) Steinbach, S.; Serschnitzki, B.; Gerlach, M.; Marcus, K.; May, C. Spiked human substantia nigra proteome data set for use as a spectral library for protein modelling and protein mapping. *Data Brief* **2019**, *23*, 103711. DOI: 10.1016/j.dib.2019.103711.