



(51) International Patent Classification:

A61B 3/02 (2006.01) G01N 33/52 (2006.01)
 C12Q 1/06 (2006.01) G01N 33/558 (2006.01)
 C12Q 1/37 (2006.01) G01N 33/577 (2006.01)
 G01N 21/64 (2006.01) G01N 33/68 (2006.01)
 G01N 21/77 (2006.01)

(21) International Application Number:

PCT/IB2017/000059

(22) International Filing Date:

13 January 2017 (13.01.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/278,805 14 January 2016 (14.01.2016) US
 62/278,814 14 January 2016 (14.01.2016) US

(71) Applicant: DIAGNOS TEAR, LTD. [IL/IL]; Kiryat Atidim, Building 3, 5th Floor, 61851 Tel Aviv (IL).

(72) Inventors: SOMMERA, Amos; 1 Oley Bavel Street, 46344 Herzliya (IL). FAKTOR, Ouriel; 1 Oley Bavel Street, 46344 Herzliya (IL). EILAT, Eran; 1 Oley Bavel Street, 46344 Herzliya (IL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

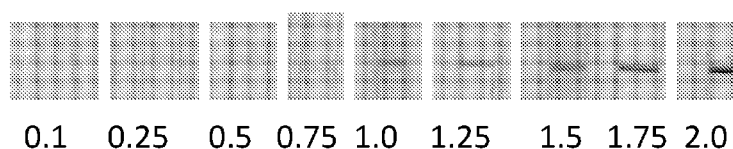
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: METHOD FOR MEASURING TEAR CONSTITUENTS IN A TEAR SAMPLE

FIGURE 1



Analyte/concentration									
HSAug/ml	0	0.01	0.025	0.05	0.1	0.3	0.5	1.2	4

(57) Abstract: In one embodiment, the present invention is a method for diagnosing Dry Eye Syndrome by quantifying an amount of at least two markers in a tear sample collected from a subject, wherein the at least two markers are selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme. In some embodiments, the method is a multi-assay test.

METHOD FOR MEASURING TEAR CONSTITUENTS IN A TEAR SAMPLE**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to U.S. Provisional Patent Application Serial Nos. 62/278,805, and 62/278,814, filed on January 14, 2016, the entire contents of which are incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] In some embodiments, the present invention is a method for quantifying an amount of at least two tear constituents in a tear sample, selected from the group consisting of lysozyme, lactoferrin, mucin, HSA, and any combination thereof. In some embodiments, the method is a multi-assay test.

SUMMARY

[0003] The following embodiments and aspects thereof are described and illustrated in conjunction with systems, tools and methods which are meant to be exemplary and illustrative, not limiting in scope.

[0004] Among those benefits and improvements that have been disclosed, other objects and advantages of this invention will become apparent from the following description taken in conjunction with the accompanying figures. Detailed embodiments of the present invention are disclosed herein; however, it is to be understood that the disclosed embodiments are merely illustrative of the invention that may be embodied in various forms. In addition, each of the examples given in connection with the various embodiments of the invention which are intended to be illustrative, and not restrictive.

[0005] Throughout the specification and claims, the following terms take the meanings explicitly associated herein, unless the context clearly dictates otherwise. The phrases "in one

embodiment" and "in some embodiments" as used herein do not necessarily refer to the same embodiment(s), though it may. Furthermore, the phrases "in another embodiment" and "in some other embodiments" as used herein do not necessarily refer to a different embodiment, although it may. Thus, as described below, various embodiments of the invention may be readily combined, without departing from the scope or spirit of the invention.

[0006] In addition, as used herein, the term "or" is an inclusive "or" operator, and is equivalent to the term "and/or," unless the context clearly dictates otherwise. The term "based on" is not exclusive and allows for being based on additional factors not described, unless the context clearly dictates otherwise. In addition, throughout the specification, the meaning of "a," "an," and "the" include plural references. The meaning of "in" includes "in" and "on."

[0007] In some embodiments, the present invention is a method for quantifying an amount of at least one marker in a tear sample, selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme, comprising: collecting the tear sample containing the amount of the at least one marker from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of the at least one marker of the tear sample is used to generate a semi-quantitative measurement of the at least one marker by: collecting the tear sample containing the amount of the at least one marker from the subject; contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of an at least one antibody specific for the at least one marker, where the amount of the at least one antibody is configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample, incubating the amount of the at least one marker

from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker; and utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker; where the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[0008] In some embodiments, the method further comprises: correlating the amount of the at least one marker in a tear sample with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[0009] In some embodiments, the semi-quantitative measurement of the at least one marker is used to calculate the probability of the subject having dry eye.

[00010] In some embodiments, the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-0.6491 - 1.1142 * Albumin)}{1 + \exp(-0.6491 - 1.1142 * Albumin)}$$

[00011] In some embodiments, the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-4.4755 - 1.9616 * Albumin - 10.2477 * Lactoferrin + 0.1263 * Age (yrs))}{1 + \exp(-4.4755 - 1.9616 * Albumin - 10.2477 * Lactoferrin + 0.1263 * Age (yrs))}$$

$$\frac{+0.3566 * (-1 \text{ if male}) + 8.7859 * Albumin * Lactoferrin}{+0.3566 * (-1 \text{ if male}) + 8.7859 * Albumin * Lactoferrin}$$

[00012] In some embodiments, the method quantifies an amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample.

[00013] In some embodiments, the method further comprises: correlating the amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample with a

measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00014] In some embodiments, the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00015] In some embodiments, the amount of mucin of the tear sample is used to generate a semi-quantitative measurement of mucin by: collecting the tear sample containing the amount of mucin from the subject; contacting the tear sample containing the amount of mucin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of Jacalin bound to biotin and an amount of wheat germ agglutinin (WGA), where the amount of the Jacalin bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ Jacalin bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of mucin from the subject on the tear analyzing strip so as to result in a line intensity of mucin; and utilizing the line intensity of mucin to determine the semi-quantitative measurement of mucin; where the semi-quantitative measurement of mucin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00016] In some embodiments, the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00017] In some embodiments, the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of

lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00018] In some embodiments, the present invention is a method for quantifying an amount of Human Serum Albumin (HSA) in a tear sample, comprising: collecting the tear sample containing the amount of HSA from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00019] In some embodiments, the method further comprises: correlating the semi-quantitative measurement of HSA with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00020] In some embodiments, the present invention is a method for quantifying an amount of mucin in a tear sample, comprising: collecting the tear sample containing the amount

of mucin from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of mucin of the tear sample is used to generate a semi-quantitative measurement of mucin by: collecting the tear sample containing the amount of mucin from the subject; contacting the tear sample containing the amount of mucin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of Jacalin bound to biotin and an amount of wheat germ agglutinin (WGA), where the amount of the Jacalin bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ Jacalin bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of mucin from the subject on the tear analyzing strip so as to result in a line intensity of mucin; and utilizing the line intensity of mucin to determine the semi-quantitative measurement of mucin; where the semi-quantitative measurement of mucin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00021] In some embodiments, the method further comprises correlating the semi-quantitative measurement of mucin with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00022] In some embodiments, the present invention provides for a method for quantifying an amount of lactoferrin in a tear sample, comprising: collecting the tear sample containing the amount of lactoferrin from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures at least 2 microliters (e.g., but not limited to, between 6 to 25 microliters), and where the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin

by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00023] In some embodiments, the method further comprises correlating the semi-quantitative measurement of lactoferrin with a measurement from the group consisting of corneal staining, Schirmer's test, and any combination thereof.

[00024] In some embodiments, the present invention is a method for quantifying an amount of lysozyme in a tear sample, comprising: collecting the tear sample containing the amount of lysozyme from a subject, where the tear sample measures at least 2 microliters, and where the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme

antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00025] In some embodiments, the method further comprises: correlating the semi-quantitative measurement of lysozyme with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00026] In some embodiments, the present invention is a device configured for performing the method for quantifying an amount of at least one marker in a tear sample, selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme.

[00027] In some embodiments, the present invention provides a method for calculating the probability of a subject having dry eye, comprising the steps of:

- a. collecting a tear sample containing at least one marker from a subject, wherein the at least one marker is selected from the group consisting of: Human Serum Albumin (HSA), lactoferrin, and lysozyme;
- b. generating a semi-quantitative measurement of the at least one marker by:
 - i. collecting the tear sample containing the amount of the at least one marker from the subject;

- ii. contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip,

wherein the tear analyzing strip contains an amount of an at least one antibody or at least one lectin specific for the at least one marker,

wherein the amount of the at least one antibody, or the at least one lectin is configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample;
- iii. incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker;
- iv. utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker,

wherein the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0; and
- v. calculating the probability of the subject having dry eye is calculated using an equation selected from the group consisting of:

1. $\frac{\exp(-0.6491-1.1142*Albumin)}{1+\exp(-0.6491-1.1142*Albumin)}$; and
2. $\frac{\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))}{1+\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))}$
 $\frac{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}$
 $\frac{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}$

[00028] In some embodiments, the present invention is a device configured to perform the method for calculating the probability of a subject having dry eye.

[00029] The figures constitute a part of this specification and include illustrative embodiments of the present invention and illustrate various objects and features thereof. Further, the figures are not necessarily to scale, some features may be exaggerated to show details of particular components. In addition, any measurements, specifications and the like shown in the figures are intended to be illustrative, and not restrictive. Therefore, specific structural and functional details disclosed herein are not to be interpreted as limiting, but merely as a representative basis for teaching one skilled in the art to variously employ the present invention. The figures are listed below.

BRIEF DESCRIPTION OF THE FIGURES

[00030] Figure 1 shows an intensity result of the test strip/tear analyzing strip to a control test strip according to some embodiments of the present invention.

[00031] Figure 2 shows a non-limiting exemplary embodiment of a Tear Analyzing Strip (TAS) according to some embodiments of the present invention.

DESCRIPTION

[00032] In some embodiments of the method of the present invention, the following is a list of terms and accompanying abbreviations of the terms used herein:

Abbreviation	Term
AE	adverse event
BCA	bicinchoninic acid, reagent for protein determination
CAE	controlled adverse environment
DE	dry eye
ETDRS	Early Treatment of Diabetic Retinopathy Study
FDA	Food and Drug Administration
G	Gram
IOP	intraocular pressure
IRB	institutional/independent review board
IU	international unit
IV	Intravenous
Kg	Kilogram
logMAR	logarithm of the minimum angle of resolution
MedDRA	Medical Dictionary for Regulatory Activities
Mg	Milligram
μg	Microgram
mL	Milliliter
μL	microliter
mm	Millimeter
μm	Micrometer
OSDI	Ocular surface disease index
PBS	Phosphate-buffered saline
TFBUT	Tear film break-up time

[00033] In some embodiments, the method of the present invention includes the use of at least one diagnostic test. In some embodiments, in performing such a comparison of tear constituents in healthy and dry eye subjects, a multiplicative effect is obtained. In some embodiments, a kit is used to provide an assessment between severe patients and healthy subjects.

[00034] In some embodiments, the present invention is a method for quantifying an amount of at least one marker in a tear sample, selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme, comprising: collecting the tear sample containing the amount of the at least one marker from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of the at least one marker of the tear sample is used to generate a semi-quantitative measurement of the at least one marker by: collecting the tear sample containing the amount of the at least one marker from the subject; contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of an at least one antibody specific for the at least one marker, where the amount of the at least one antibody is configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample, incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker; and utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker; where the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00035] In some embodiments, the method further comprises: correlating the amount of the at least one marker in a tear sample with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00036] In some embodiments, the present invention provides a method for diagnosing Dry Eye Syndrome by quantifying an amount of at least two markers in a tear sample collected from a subject, wherein the at least two markers are selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme, comprising:

- a. collecting the tear sample containing the amount of the at least one marker from a subject;
- b. generating a semi-quantitative measurement of the at least one marker by:
 - i. collecting the tear sample containing the amount of the at least one marker from the subject;
 - ii. contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip,

wherein the tear analyzing strip contains an amount of an at least one antibody, or at least one lectin specific for the at least one marker,

wherein the amount of the at least one antibody, or the at least one lectin are configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample;

- iii. incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker; and
- iv. utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker,
 - wherein the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00037] In some embodiments, the method quantifies an amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample. In some embodiments, the volume of the tear sample is between 0.1 and 25 microliters.

[00038] In some embodiments, the method further comprises: correlating the amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00039] In some embodiments, the patient is diagnosed as having dry eye disease when the corneal staining measurement exceeds a predetermined measurement, the Schirmer's test measurement exceeds predetermined measurement, the OSDI measurement exceeds predetermined measurement, or any combination thereof.

[00040] As used herein, the term "dry eye disease" refers a disorder of the tear film resulting from tear deficiency which causes discomfort and damage to the inter-palpebral ocular surface. In some embodiments of the method of the present invention, the dry eye disease can be

caused by, but not limited to, exacerbation by environmental conditions, by lifestyle choices, or by other medications.

[00041] As used herein, the term "effective volume," when used to describe tears collected in some methods of the embodiments of the invention disclosed herein, refers to a volume large enough to provide a definitive result when subjected to a particular chemical or physical test. Thus, the "effective volume" will depend on the particular test being performed.

[00042] As used herein, the term "mild dry eye" refers to transient symptoms or signs of the disease that do not require treatment, as diagnosed by a patient and/or a medical professional (e.g., but not limited to, a doctor, a nurse, etc.). For dry eye to be considered moderate, patients must experience signs or symptoms that are responsive to simple therapeutic measures (e.g., but not limited to, applying eye drops to the dry eye(s)).

[00043] As used herein, the term "semi-quantitative measurement" refers to a result obtained from an assay, where the assay includes a fixed running time and use of a test strip(s) configured to receive a tear containing at least one tear constituent (e.g., total protein) by a medical professional, and where a medical professional reads and compares an intensity result of the test strip/tear analyzing strip to a control test strip (e.g., as shown in Figure 1) containing a plurality of line intensities so as to determine whether the intensity result of the test strip indicates that a subject has a dry eye disease. In some embodiments, this semi-quantitative measurement can be used for comparison and correlation to other tests, such as the Schirmer's test, TFBUT, OSDI, corneal staining, or any combination thereof.

[00044] As used herein, the term "tear(s)" refer(s) to an extracellular fluid covering the surface epithelial cells of the corneal and conjunctival epithelium, where the tear film represents the last line of defense for the ocular surface. The primary functions of the tear film are to

lubricate the surface and the lids, to optimize the refractive function of the anterior segment, and to provide a means for removal of environmental contaminants from the ocular surface. The normal tear film is composed of three layers: an outer lipid layer (approximately 0.1 μm thick) produced by the meibomian glands in the tarsal plate, a central aqueous layer (approximately 7 to 10 μm thick) produced by both the main and accessory lacrimal glands, and an inner mucin layer (approximately 0.2 to 1.0 μm thick) produced by goblet cells in the conjunctiva.

[00045] As used herein, the term "tear components" refer to the molecules in tears and includes, but is not limited to, water, electrolytes, antimicrobial molecules, immunoglobulins, lipids, growth factors, or any combination thereof. When the quality or quantity of tears is compromised by an imbalance or breakdown in any of these components, the result can be a cause or exacerbation of dry eye symptoms.

[00046] As used herein, the term "human serum albumin" or "HSA" is a major tear protein and can serve as gross-index for total protein. As used herein, the term "Bicinchoninic acid" or "BCA" refers to a method used for total protein determination; it is known for both a high sensitivity and low frequency of interference from extraneous non-proteinaceous substances.

[00047] As used herein, the term "mucin" refers to mucin(s) found in tears. Mucins are complex proteoglycans that include both soluble and epithelial surface forms, and both provide a lubrication function for the ocular surface. Soluble tear mucins are primarily secreted by conjunctival goblet cells. Total mucin levels can be measured by assays of branched-chain carbohydrate content.

[00048] As used herein, the term "lactoferrin" refers to a protein synthesized and secreted by the acini of the lacrimal gland. The amount of lactoferrin present in normal tears ranges from

0.6-3.0 mg/ml, where it acts as an antibacterial by reducing free iron and a free radical scavenger.

[00049] As used herein, the term "lysozyme" refers to a protein synthesized and secreted by the acini of the lacrimal gland. The amount of lysozyme present in normal tears ranges from 0.6-2.6 mg/ml, where it acts as an antibacterial by degrading cell wall components of bacteria in the tear film.

[00050] According to some embodiments, the method of the present invention includes using a Tear Analyzing Strip (TAS). Figure 2 shows a non-limiting exemplary embodiment of the TAS. Figure 3 shows a non-limiting exemplary embodiment of the TAS. In some embodiments, the TAS comprises one or more pads containing chemical or biological reagents which, upon contact with tears, undergo an immuno-chemical recognition with the tested analyte and migration of the complex to a defined zone. As a result of which, a colored line is observed. The diagnosis may be made after a predefined time, e.g. after completion of the immuno-chemical reaction. In some embodiments, the diagnosis is based on comparing the color intensity of the observed line on the TAS reaction zone to a reference printed picture color line intensities. The printed picture of color line intensities wherein each of the color intensity represent one or more characteristics for diagnosing DES. Such characteristics may be, but not limited to, (a) the concentration of at least one substance the concentration of which is known to correlate with DES (a non-limiting example includes HSA), the concentration of at least one predefined protein level and electrolyte (such as sodium, potassium etc.) (b) osmolarity, (c) viscosity and surface tension and (d) pH.

[00051] In some embodiments of the method of the present invention, the TAS can also be useful for collecting an amount of tear fluid sufficient for performing a medical diagnosis based

on the relevant characteristics of the tears. In some embodiments, the TAS thus can provide qualitative (e.g., but not limited to, using a strip reader), quantitative, semi-quantitative and multi-factorial diagnosis. In some embodiments, the TAS thus can provide a semi-quantitative diagnosis.

[00052] In some embodiments of the method of the present invention, a Schirmer's test provides for a quantitative assessment of tear production. In some embodiments, the Schirmer's test is a measure of dry eye.

[00053] In some embodiments of the method of the present invention, a "tear film break-up time" (TFBUT) test can be used to measure and assess dry eye disease. In some embodiments of the method of the present invention, staining of corneal and conjunctival epithelial cell damage can be used to measure and assess dry eye disease. In some embodiments, symptoms of dry eye disease are variable, but quantitative assessments typically employ questionnaires such as the Ocular Surface Disease Index ("OSDI").

[00054] In some embodiments, the method of the present invention includes providing two lectins, e.g., pisum sativum agglutinin ("PSA") and lens culinaris agglutinin ("LCA"), where PSA is conjugated to gold particles. In some embodiments, biotin is bound to PSA which generates biotin-PSA, and biotin-PSA is bound to streptavidin-gold conjugate. In some embodiments, the lectins are placed on a test strip. In some embodiments, at least one lectin is conjugated to gold particles ("immunogold labeled"). In some embodiments, the gold particles are colloidal gold particles. In some embodiments, the colloidal gold particles can range from 20 to 125 nm. In some embodiments, the colloidal gold particles can range from 50 to 125 nm. In some embodiments, the colloidal gold particles can range from 100 to 125 nm. In some embodiments, the colloidal gold particles can range from 20 to 100 nm. In some embodiments,

the colloidal gold particles can range from 20 to 50 nm. In some embodiments, the colloidal gold particles can range from 20 to 60 nm. In some embodiments, the colloidal gold particles can range from 20 to 40 nm. In some embodiments, the colloidal gold particles can range from 40 to 60 nm. In some embodiments, the colloidal gold particles can range from 50 to 100 nm.

[00055] In some embodiments, the test strip contains Nitrocellulose (e.g., but not limited to, Whatman's FF120 or the CNPH-N-SS60 from Advanced Microdevices PVT).

[00056] In some embodiments, the method of the present invention includes a comparative step where the semi-quantitative intensity measurement of total protein is correlated with results of the Schirmer's method. According to Schirmer's method, a paper strip is used to measure the amount of tears produced over a period of five minutes. The strip is placed at the junction of the middle and lateral thirds of the lower eyelid, between the eyeball and the lid. The test is done under ambient light. The patient is instructed to look forward and to blink normally during the course of the test. Wetting of more than 10 mm of the paper in 5 minutes is taken to indicate that the eye produces normal quantity of tears. The specificity (the ability of the test to identify negative results) of Schirmer method is usually around 90%. The Schirmer test provides true positive results when the wetting is less the 5 mm and true negative results when the level of wetting is above 10 mm and may provide false positive results when the level of wetting is between 5 mm and 10 mm. When the level of wetting is between 5 mm and 10 mm the patient is suspected to have DES, but the results cannot be considered conclusive.

[00057] In some embodiments, the method of the present invention includes a comparative step where the semi-quantitative intensity measurement of mucin is correlated with results of the Schirmer's method. According to Schirmer's method, a paper strip is used to measure the amount of tears produced over a period of five minutes. The strip is placed at the junction of the middle

and lateral thirds of the lower eyelid, between the eyeball and the lid. The test is done under ambient light. The patient is instructed to look forward and to blink normally during the course of the test. Wetting of more than 10 mm of the paper in 5 minutes is taken to indicate that the eye produces normal quantity of tears. The specificity (the ability of the test to identify negative results) of Schirmer method is usually around 90%. The Schirmer test provides true positive results when the wetting is less the 5 mm and true negative results when the level of wetting is above 10 mm and may provide false positive results when the level of wetting is between 5 mm and 10 mm. When the level of wetting is between 5 mm and 10 mm the patient is suspected to have DES, but the results cannot be considered conclusive.

[00058] In some embodiments, the method of the present invention includes a comparative step where the semi-quantitative intensity measurement of lactoferrin is correlated with results of the Schirmer's method. According to Schirmer's method, a paper strip is used to measure the amount of tears produced over a period of five minutes. The strip is placed at the junction of the middle and lateral thirds of the lower eyelid, between the eyeball and the lid. The test is done under ambient light. The patient is instructed to look forward and to blink normally during the course of the test. Wetting of more than 10 mm of the paper in 5 minutes is taken to indicate that the eye produces normal quantity of tears. The specificity (the ability of the test to identify normal individuals) of Schirmer method is usually around 90%. The Schirmer test provides true identification of DED suspected individuals at a rate of 20% of total DED suspected population. The Schirmer test provides true positive results when the wetting is less the 5 mm and true negative results when the level of wetting is above 10 mm and may provide false positive results when the level of wetting is between 5 mm and 10 mm. When the level of wetting is between 5

mm and 10 mm the patient is suspected to have DES, but the results cannot be considered conclusive.

[00059] In some embodiments, the method of the present invention includes a comparative step where the semi-quantitative intensity measurement of lysozyme is correlated with results of the Schirmer's method. According to Schirmer's method, a paper strip is used to measure the amount of tears produced over a period of five minutes. The strip is placed at the junction of the middle and lateral thirds of the lower eyelid, between the eyeball and the lid. The test is done under ambient light. The patient is instructed to look forward and to blink normally during the course of the test. Wetting of more than 10 mm of the paper in 5 minutes is taken to indicate that the eye produces normal quantity of tears. The specificity (i.e., the ability of the test to identify normal individuals) of Schirmer method is usually around 90%. The Schirmer test provides true identification of DED suspected individuals - at a rate of 20% of total DED suspected population. The Schirmer test provides true positive results when the wetting is less the 5 mm and true negative results when the level of wetting is above 10 mm and may provide false positive results when the level of wetting is between 5 mm and 10 mm. When the level of wetting is between 5 mm and 10 mm the patient is suspected to have DES, but the results cannot be considered conclusive.

[00060] In some embodiments, the semi-quantitative measurement of the at least one marker is used to calculate the probability of the subject having dry eye.

[00061] In some embodiments, the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-0.6491 - 1.1142 * \text{Albumin})}{1 + \exp(-0.6491 - 1.1142 * \text{Albumin})}$$

[00062] In some embodiments, after calculating this probability, one then assigns a subject to a group (dry eye or healthy) based on the probability. Using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye $34/44 = 77.4\%$ of time and correctly classifies healthy subjects as healthy $9/30 = 30.0\%$ of the time.

[00063] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $30/44 = 68.2\%$ of time and correctly classifies healthy subjects as healthy $19/30 = 63.3\%$ of the time.

[00064] In some embodiments, the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-4.4755 - 1.9616 * \text{Albumin} - 10.2477 * \text{Lactoferrin} + 0.1263 * \text{Age (yrs)})}{1 + \exp(-4.4755 - 1.9616 * \text{Albumin} - 10.2477 * \text{Lactoferrin} + 0.1263 * \text{Age (yrs)})}$$

$$\frac{+0.3566 * (-1 \text{ if male}) + 8.7859 * \text{Albumin} * \text{Lactoferrin}}{+0.3566 * (-1 \text{ if male}) + 8.7859 * \text{Albumin} * \text{Lactoferrin}}$$

[00065] In some embodiments, the present invention provides a method for calculating the probability of a subject having dry eye, comprising the steps of:

- a. collecting a tear sample containing at least one marker from a subject, wherein the at least one marker is selected from the group consisting of: Human Serum Albumin (HSA), lactoferrin, and lysozyme;
- b. generating a semi-quantitative measurement of the at least one marker by:
 - i. collecting the tear sample containing the amount of the at least one marker from the subject;
 - ii. contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip,

wherein the tear analyzing strip contains an amount of an at least one antibody or at least one lectin specific for the at least one marker,

wherein the amount of the at least one antibody, or the at least one lectin is configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample;

iii. incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker;

iv. utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker,

wherein the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0; and

v. calculating the probability of the subject having dry eye is calculated using an equation selected from the group consisting of:

1. $\frac{\exp(-0.6491-1.1142*Albumin)}{1+\exp(-0.6491-1.1142*Albumin)}$; and
2. $\frac{\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))}{1+\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))} + \frac{0.3566*(-1 \text{ if male})+8.7859*Albumin*Lactoferrin}{0.3566*(-1 \text{ if male})+8.7859*Albumin*Lactoferrin}$.

[00066] In some embodiments, after calculating this probability, one then assigns a subject to a group (dry eye or healthy) based on the probability. Using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye 39/44 = 88.6% of time and correctly classifies healthy subjects as healthy 23/30 = 76.7% of the time.

[00067] Increasing the cutoff probability to 55%, the model correctly classifies dry eye subjects as having dry eye $37/44 = 84.1\%$ of time and correctly classifies healthy subjects as healthy $24/30 = 80.0\%$ of the time.

[00068] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $36/44 = 81.8\%$ of time and correctly classifies healthy subjects as healthy $26/30 = 86.7\%$ of the time.

[00069] The results of this model show that choosing either a cutoff probability of 55% or 60% yield sensitivity and specificity $\geq 80\%$.

[00070] In some embodiments, using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye $40/44 = 90.9\%$ of time and correctly classifies healthy subjects as healthy $23/30 = 76.7\%$ of the time.

[00071] Increasing the cutoff probability to 55%, the model correctly classifies dry eye subjects as having dry eye $38/44 = 86.4\%$ of time and correctly classifies healthy subjects as healthy $26/30 = 86.7\%$ of the time.

[00072] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $36/44 = 81.8\%$ of time and correctly classifies healthy subjects as healthy $27/30 = 90.0\%$ of the time.

[00073] In some embodiments, the addition of lysozyme and the interactions of lysozyme*albumin and lysozyme*lactoferrin improves the sensitivity and specificity slightly at each cutoff probability.

The at Least One Marker

[00074] *Human Serum Albumin:* In some embodiments, the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear

sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00075] In some embodiments, the present invention is a method for quantifying an amount of Human Serum Albumin (HSA) in a tear sample, comprising: collecting the tear sample containing the amount of HSA from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00076] In some embodiments, the method further comprises: correlating the semi-quantitative measurement of HSA with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00077] *Mucin:* In some embodiments, the amount of mucin of the tear sample is used to generate a semi-quantitative measurement of mucin by: collecting the tear sample containing the amount of mucin from the subject; contacting the tear sample containing the amount of mucin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of Jacalin bound to biotin and an amount of wheat germ agglutinin (WGA), where the amount of the Jacalin bound to biotin is conjugated to colloidal gold at a ratio of 5 µg/ml Jacalin bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of mucin from the subject on the tear analyzing strip so as to result in a line intensity of mucin; and utilizing the line intensity of mucin to determine the semi-quantitative measurement of mucin; where the semi-quantitative measurement of mucin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00078] In some embodiments, the present invention is a method for quantifying an amount of mucin in a tear sample, comprising: collecting the tear sample containing the amount of mucin from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures between at least 2 microliters (e.g., but not limited to, 6 to 25 microliters), and where the amount of mucin of the tear sample is used to generate a semi-quantitative measurement of mucin by: collecting the tear sample containing the amount of mucin from the subject; contacting the tear sample containing the amount of mucin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount

of Jacalin bound to biotin and an amount of wheat germ agglutinin (WGA), where the amount of the Jacalin bound to biotin is conjugated to colloidal gold at a ratio of 5 µg/ml Jacalin bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of mucin from the subject on the tear analyzing strip so as to result in a line intensity of mucin; and utilizing the line intensity of mucin to determine the semi-quantitative measurement of mucin; where the semi-quantitative measurement of mucin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00079] In some embodiments, the method further comprises correlating the semi-quantitative measurement of mucin with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00080] *Lactoferrin:* In some embodiments, the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 µg/ml PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative

measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00081] In some embodiments, the present invention provides for a method for quantifying an amount of lactoferrin in a tear sample, comprising: collecting the tear sample containing the amount of lactoferrin from a subject by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface, where the tear sample measures at least 2 microliters (e.g., but not limited to, between 6 to 25 microliters), and where the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00082] In some embodiments, the method further comprises correlating the semi-quantitative measurement of lactoferrin with a measurement from the group consisting of corneal staining, Schirmer's test, and any combination thereof.

[00083] *Lysozyme*: In some embodiments, the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00084] In some embodiments, the present invention is a method for quantifying an amount of lysozyme in a tear sample, comprising: collecting the tear sample containing the amount of lysozyme from a subject, where the tear sample measures at least 2 microliters, and where the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme

antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

[00085] In some embodiments, the method further comprises: correlating the semi-quantitative measurement of lysozyme with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.

[00086] The present invention is further illustrated, but not limited by, the following examples.

EXAMPLES

Example 1: Measurement of Human Serum Albumin in Tear Samples

[00087] The levels of a prominent tear constituent was examined in healthy subjects and in subjects who met one or more criteria of mild to moderate dry eye. The following experiments illustrate a comparison between benchmark testing for assessment of dry eye with a quantitative measure of a tear constituent. Examples of the tests used to quantitatively measure at least one tear constituent are corneal staining, Schirmer's tests, TFBUT, and provided symptom assessment including the OSDI questionnaire and the Ora-CalibraTM ocular discomfort score. The OSDI is a 12 question assessment that has become a standard for dry eye symptomology. The Ora-Calibra assessments for discomfort also provide a measurement of

symptomatology by allowing a patient to answer questions, where the number of questions is reduced compared to the OSDI. Samples of tears were collected using capillary tubes and then underwent analysis for the tear constituent. The tear constituent measured was total protein.

Tear constituent assay and measurement methodology:

[00088] Rapid test strips/TAS (i.e., tear analyzing strips) and reagents were used to measure HSA levels using a semi-quantitative technique; where the semi-quantitative technique followed a fixed running time for each type of assay, strips were scanned with HP's scanner model scanjet 200. The resulting scanned figure was optimized using Function Lighten / Darken: Highlights – (-) 50; Shadows – (-) 69; Midtones – (-) 50; Gamma- 1.7 followed by recording of signal intensity (as shown in Figure 1). Determination of the tear constituent was conducted using semi-quantitative estimation of the intensity test lines compared to intensity of a printer picture of color intensities.

Experimental Design:

Subject Population

[00089] Subjects for the study included anyone over the age of 18 years who met the inclusion and exclusion criteria listed in the following tables. The study population included two groups of subjects (Group A, as shown in Table 1, and Group B, shown in Table 2) with approximately equal numbers of each (~ 100 subjects per group):

Table 1: Group A – Healthy Eyes

<i>Healthy Subjects, Inclusion Criteria</i>	
1.	Subject must be 18 years of age and may be of any race and either gender;
2.	The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3.	Subject agrees for samples to be taken from both eyes;
4.	Subject must be willing to follow the study procedures and visit schedule;
5.	Subject must report <2 in all symptoms (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
6.	Subject has at least one of the following in the collection eye(s): <2 in all regions of the cornea (Ora Calibra™ Scale) during visit; - TFBUT > 10 seconds during visit.
<i>Healthy Subjects, Exclusion Criteria</i>	
1.	Subject complained of dry eye or any other acute ocular disease ;
2.	Subject is currently suffering from active inflammation or infection;
3.	Subject used artificial tear drops in the past 2 months;
4.	Subject currently treated medically for a chronic eye syndrome such as glaucoma, allergy or conjunctivitis;
5.	Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or which would present a special risk to the subject;
6.	Subject reports currently being pregnant or nursing;
7.	Use of investigational study drug or study device within 30 days of enrollment.

Table 2: Group B – Suspected Dry Eye

<i>Suspected Dry Eye, Inclusion Criteria</i>
1. Subject must be 18 years of age and may be of any race and either gender;
2. The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3. Subject used or had the desire to use artificial tears in the last 30 days;
4. Subject reports ≥ 2 in at least one symptom (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
5. Subject demonstrates both of the following in the collection eye(s): a. ≥ 2 in at least one region (Ora Calibra™ scale) b. TFBUT < 10 seconds during visit;
6. Subject agrees for samples to be taken from both eyes;
7. Subject must be willing to follow the study procedures and visit schedule.
<i>Suspected Dry Eye, Exclusion Criteria</i>
1. Subject is using contact lenses on a regular basis;
2. Subject is currently suffering from active inflammation or infection;
3. Subject has used Restasis® in the last 30 days;
4. Subject used artificial tear drops in the past hour;
5. Subject is being medically treated for glaucoma;
6. Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or would present a special risk to the subject;
7. Subject reports currently being pregnant or nursing;
8. Subject has participated in any other clinical trial within 30 days of enrollment.

Experimental Design and Methods

[00090] An exemplary embodiment of the method of the present invention was a prospective, single-center, single-visit, parallel-group, data and tear collection study, consisting of approximately 200 subjects. There was one scheduled study visit where subjects were screened; those who met the eligibility criteria were enrolled in the study.

Tear Sample Collection

[00091] The procedure for tear sample collection was as follows:

1. The slit-lamp was set at a low intensity beam.
2. The lower lid of the eye was retracted and a glass capillary tube was placed on the temporal aspect touching the tear surface.
3. The tear surface was contacted and allowed for the collection of between 6 to 25 microliters of tear solution.
4. Once a sufficient volume (e.g., but not limited to, 6 to 25 microliters) was collected, the contents of the glass capillary was withdrawn and emptied into a vial. If tear volume was below 6 microliters, a second sample was drawn from the other eye into another clean vial.
5. The vials were marked with a designated subject label provided by the sponsor.
6. The vials were stored at a temperature of 2°C-8°C. Tear samples were transferred to the sponsor laboratories for initial preparation up to 48 hours from collection before further analysis for levels of total protein.
7. The tear volume was measured within the 48 hours from sampling using pipette of small volume. Two sample volumes of Phosphate buffer saline (PBS X 1) were added to

collected sample followed by a short vortex (20 Sec.) for mixing. Diluted samples were placed back for storage in a temperature of 2°C-8°C.

Total Protein Assay

[00092] This assay allows for the detection of HSA in human tears by using monoclonal antibodies that recognize the molecule. The test strip is based on semiquantitative lateral flow immunochromatographic technology. First, a diluted tear sample was placed on the sample pad. Then, additional drops of washing solution were placed on the sample pad to allow the tear sample to migrate and wet the conjugate pad. Specific Mouse monoclonal antibodies to HSA conjugated to gold particles bind the Albumin. The conjugated antibodies with bound Albumin flow through the nitrocellulose (NC) membrane. When the gold conjugate/HSA complex reaches the test zone on the NC membrane it reacts with a second monoclonal anti HSA antibody fixated to the membrane surface. Together they form a pinkish red visible line. An excess amount of complex then proceeds to a second zone where anti mouse IgG are impregnated and bind the monoclonal anti HSA-gold conjugate. A second line is formed (Control line). The control line indicates of test validity. Residual amount of conjugate and tear sample clear out from the NC membrane into the sink pad. Figure 2 shows an example of the semiquantitative lateral flow immunochromatographic technology.

[00093] Two sets of monoclonal antibodies are used. Each of the clones binds specific nonidentical epitope on the HSA molecule. 1mg/ml (can range between 0.75 and 2.5 mg/ml) monoclonal anti HSA is impregnated onto a chromatographic membrane of Nitrocellulose (Whatman FF120). Impregnation is in the shape of a 1mm wide line. The antibody solution also contains the following: (a) Buffer, for example, Phosphate buffer saline at pH 7.4 or possibly Tris, HE PES, Borax or MES buffer with pH value ranging from 6.5 to 9.0; (b) 2% Trehalose

(can also be Sucrose), can also range between 1% to 4% sugar; (c) 2% Ethanol, can also range from 1 to 4%, or any combination thereof. Antibody impregnated NC is dried at SOC for 10 Min in order to fixate the protein to the NC. Fixation can also be done between 60°C and 37°C for 5 minutes to 24 hours depending on the temperature. Second clone of Mouse anti HSA is conjugated to gold particles (40 nm of size can also be 20nm or 60 nm) at a ratio of 2µg protein per 001 per ml colloidal gold at 528nm. Conjugation is done under pH of 9 (can be also done under pH 7 or 8). Gold conjugate is blocked with BSA and PEG (15-20K). Effective Concentration of the gold conjugate can range from 000.5 /ml to OD 2/ml. Line intensity is estimated visually (e.g. Figure 1), and the line intensity of 1 is formed when the strip detects 1.2 µg/ml HSA.

[00094] Reaction mix can also include Wash Reagent (WR) that provides chemical surrounding as well as clearing of gold residuals from the NC membrane. The WR contains the following: (a) PBS X 1 pH 7.4 (can range from 7 to 9); (b) 1% BSA can range from 0.5 to 3% and should be fatty acid free. If not purified enough we get NSB due to unwanted sugar groups; (c) 0.1% Tween 20 (can range from 0.05% to 2%), (d) 0.05% N-lauroyl sarcosine (i.e., may range from between 0.01 - 1.0%) and 0.4% PEG; or any combination thereof.

Tear Film Break Up Time Test

[00095] The procedure for TFBUT included:

1. A medical professional instilled 5 µL of 2% preservative-free sodium fluorescein solution into the inferior conjunctival cul-de-sac of each eye. To thoroughly mix the fluorescein with the tear film, the subject was instructed to blink several times. In order to achieve maximum fluorescence, the medical professional waited approximately 30 seconds after instillation before evaluating TFBUT.

2. With the aid of a slit lamp, the medical professional monitored the integrity of the tear film, noting the time it takes to form micelles from the time that the eye was opened. TFBUT was measured in seconds using a stopwatch and a digital image recording system for the right eye followed by the left eye. A Wratten #12 yellow filter was used to enhance the ability to grade TFBUT.

3. For each eye, two measurements were taken and averaged unless the two measurements were greater than 2 seconds apart and were each less than 10 seconds, in which case, a third measurement was taken and the two closest of the three was averaged.

Corneal Fluorescein Staining:

[00096] The procedure for corneal fluorescein included:

1. In order to achieve maximum fluorescence, the medical professional waited approximately 3-5 minutes after instillation before evaluating fluorescein staining. A Wratten #12 yellow filter was used to enhance the ability to grade fluorescein staining.
2. The inter-palpebral was graded and recorded, and the conjunctiva and cornea epithelial were stained by use of a 5 point scale. The upper eyelid was lifted slightly to grade the whole corneal surface. Regarding conjunctiva, temporal zone grading was performed when the subject looks nasally; grading nasally by looking temporally.
3. The conjunctival and corneal staining was graded and recorded using the Ora Calibra™ Corneal and Conjunctival Staining Scale.

Unanesthetized Schirmer's Test

[00097] The Schirmer Tear Test was performed according to the following procedure:

1. Using a sterile Tear Flo Schirmer's test strip (e.g., obtained from, but not limited to, Rose Enterprises), a bend in the strip was made in line with the notch in the strip.

2. The subject will be instructed to gaze up and in.
3. The Schirmer's test strip was placed in the lower temporal lid margin of each eye such that the strip fits tightly. Subjects were instructed to close their eyes.
4. After 5 minutes have elapsed, the Schirmer's strip was removed. The length of the moistened area was recorded (mm) for each eye.

Ora Calibra™ Ocular Discomfort Scale

[00098] In an exemplary embodiment, ocular discomfort scores were subjectively graded by the subjects according to the following scale, rating each eye separately. The scale used is shown below and ranges from 0-4:

0 = no discomfort

1 = intermittent awareness

2 = constant awareness

3 = intermittent discomfort

4 = constant discomfort

Ora Calibra™ Ocular Discomfort & 4-Symptom Questionnaire

[00099] Subjects rated the severity of each of the following symptoms, with regards to how both their eyes felt, in general – overall ocular discomfort, burning, dryness, grittiness and stinging according to the following 6-point (0 to 5) scale where 0 = none and 5 = worst.

0	1	2	3	4	5
(None)					(Most)

[000100] Standards of professional care to protect the ocular safety of subjects were followed with regard to study regimen adherence. Subjects who met entry criteria provided demographic information, medical and ocular history and artificial tears use if appropriate. Clinical staff confirmed that subjects did not use artificial tears in the hour prior to the study, then guided subjects through the following procedures:

- (1) Subjects completed the OSDI© questionnaire and Ora Calibra™ Ocular Discomfort & 4-symptom Questionnaire.
- (2) Subjects and staff reviewed source documents to confirm that subject met all inclusion/exclusion criteria based on current medications and medical history.
- (3) Clinical staff collected 6-25 microliters of tears using a capillary from the right eye of the subject. Staff labeled the collection vial with the subject screening number and emptied the capillary contents into the vial.
- (4) In the cases where tear volume collected from the right eye was below 6 microliters, a sample was drawn from the left eye and the capillary was emptied into another clean vial marked with same subject screening number.

- (5) Clinical staff performed tear film break up time test on collection eye(s).
- (6) Clinical staff performed Corneal Fluorescein Staining and examined the ocular surface of the collection eye(s).
- (7) If 6 or greater microliters were collected from the right eye, but the subject did not meet Tear Film Break Up Time or Fluorescein Staining inclusion criteria in the right eye, steps 3-6 were repeated in left eye.
- (8) Clinical staff performed un-anesthetized Schirmer's Test on collection eye(s).
- (9) Clinical staff reviewed results to determine if patient met all inclusion/exclusion criteria based on data collected according to items 3-8.
- (10) Patients who met all criteria were assigned a subject study number and categorized on label based on diagnosis of healthy or suspected dry eye patient.
- (11) Adverse events, if applicable, were documented.

[000101] Samples were handled and tested using the following parameters:

- (1) Volume of collected tears was measured using a micropipette. Twice the measured volume was added with Phosphate Buffer Saline (PBS) for a final dilution of 1:3.
- (2) Diluted tears were further diluted serially to the following dilutions: 1:50, 1:100 and 1:200 with PBS.
- (3) Two microliters of diluted sample were mixed with 18 microliters of gold conjugate mix in a microtube. The relevant test strip was dipped in that mix for 4 minutes.
- (4) Additional 25 microliters of wash solution were added to the tube for excess dye clearance from reaction zone.
- (5) After 6 minutes developed test strips were gently blot against tissue paper and scanned with a desk scanner.

(6) Test intensity was quantified according to the intensity scale presented in Figure 2.

Power Analysis:

[000102] Table 3 presents power for selected sample sizes.

Table 3:

Performance Goal	Sample Proportion	N	Power
0.55	0.70	95	0.82
	0.72	75	0.82
	0.75	50	0.84
0.60	0.70	100	0.55
	0.72	100	0.71
	0.75	90	0.84
0.65	0.70	100	0.16
	0.72	100	0.29
	0.75	100	0.55

[000103] The power was estimated using Exact Binomial method, where two co-primary endpoints (sensitivity and specificity) were taken into account, and where “N” represents the number of positive only (or negative only) cases. Thus, the total sample size was doubled.

[000104] Table 4 illustrates a “Precision” parameter, which is defined as a half-length of confidence interval (CI). The CI is an interval estimate of a population parameter. The CI is an observed interval (i.e. it is calculated from the observations), in principle different from sample to sample, that frequently includes the parameter of interest if the experiment is repeated.

Table 4: Confidence Interval Precision

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
80	48	60.0%	48.4%	70.8%	11.2%
	52	65.0%	53.5%	75.4%	11.0%
	56	70.0%	58.7%	79.8%	10.6%
	60	75.0%	64.0%	84.1%	10.1%
	64	80.0%	69.5%	88.2%	9.4%
	68	85.0%	75.2%	92.1%	8.5%
	72	90.0%	81.2%	95.6%	7.2%
	76	95.0%	87.1%	98.8%	6.1%
90	54	60.0%	49.1%	70.2%	10.6%
	59	65.6%	54.7%	75.3%	10.3%
	63	70.0%	59.4%	79.3%	10.0%
	68	75.6%	65.3%	84.1%	9.4%
	72	80.0%	70.2%	87.7%	8.8%
	77	85.6%	76.5%	92.1%	7.8%
	81	90.0%	81.8%	95.4%	6.8%
	86	95.6%	88.1%	99.1%	5.9%
100	60	60.0%	49.7%	69.7%	10.0%
	65	65.0%	54.8%	74.3%	9.8%
	70	70.0%	60.0%	78.8%	9.4%
	75	75.0%	65.3%	83.2%	9.0%
	80	80.0%	70.8%	87.4%	8.3%
	85	85.0%	76.4%	91.4%	7.5%
	90	90.0%	82.3%	95.1%	6.4%

Results and Analysis

[000105] The primary outcome of the study was the comparison of benchmark tests for dry eye such as TFBUT, Corneal staining, Schirmer's test, and OSDI questionnaires with results from a test of tear film constituents (e.g., total protein).

[000106] All collected samples obtained from patients' eyes which met the entry criteria were included in the analyses. The goal of the study was to develop an assessment tool to compare benchmark tests for dry eye with a kit that tests the tear film compound, a measurement

of total protein. Data was distributed from lowest to highest values and compared with other parameters to identify positive and negative correlations. Figure 2 illustrates the correlation of test line intensity with analyte concentration. In some embodiments, a reduced test line intensity correlates with a test for dry eye (e.g., Schirmer's test, corneal staining, OSDI, etc.).

[000107] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by Schirmer's test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by Schirmer's test.

[000108] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by a corneal staining test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by a corneal staining test.

[000109] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by OSDI. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., HSA; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by OSDI.

[000110] Sample size in this pilot study (200 total eyes, 100 per group) was not based on any power analysis, but was based on an approximation of the number of eyes sufficient to build a model for a distinguishing between healthy and suspected dry eye tears and evaluation of benchmark standard testing with the different tested parameters.

[000111] Adverse Events (AEs) included any events reported over the course of the tear collection and ocular surface assessment procedures. This clinical study involved TFBUT, corneal staining and the collection of tears for the constituent analysis. During these tests the participant may have felt a foreign body sensation. During the tear collection there may have been cases of direct contact with the eye due to movement, resulting in corneal abrasion, or eye redness. Any such events were noted and graded as follows:

- Mild: Sign or symptom, usually transient, requiring no special treatment, generally not interfering with usual activities.
- Moderate: Sign or symptom, which may be ameliorated by simple therapeutic measures; may interfere with usual activity.
- Severe: Sign or symptom that are intense or debilitating and that interfere with usual activities. Recovery was usually aided by therapeutic measures.

Results

[000112] A total of 198 subjects completed the study, including 126 women and 72 men. The breakdown of subjects according to entry criteria A or B is outlined in Table 5 below. Those who met entry criteria were not matched for age or gender in this study.

Table 5:

	Men	Women	mean age
Group A Healthy	41	59	45.5
Group B Suspected DE	31	67	58.6

Analysis

[000113] Subjects enrolled in each study group met the entry criteria of either healthy or suspected dry eye. The only demographic criteria that showed a significant difference between the two groups was age; preliminary analysis showed no significant difference in any tear metrics between the two groups. In addition, both groups displayed a range of values for the benchmark testing parameters. Based upon this observation, all subjects were pooled into a single group and analyzed using population quartiles with an assumption that the population sampled represented a continuum of dry eye severity. Using this concept, measurements for each of the benchmark tests were ranked, and mean values for each of 4 quartiles were compared to measures for the tear diagnostics.

Quartile Analysis

[000114] The quartile analysis for TFBUT, inferior staining, and Schirmer's tests are summarized in Table 6. The focus of this approach was on the extremes, quartiles 1 and 4, as these represent those patients with the largest differences for each metric. In all three measures, Q1 was the quartile with values expected for normal patients and Q4 was the quartile with values associated with dry eye disease. For example, those in Q1 have a mean TFBUT of 12.80 seconds and so would be considered normal while those in Q4 have a mean TFBUT of 2.34 seconds, consistent with a diagnosis of moderate dry eye disease. When the mean values for tested parameters in each of the TFBUT-defined quartiles and/or corneal staining were compared, associations between the break-up time metric and tear constituent dynamics emerged. The decrease in TFBUT between Q1 and Q4 is accompanied by increases in total protein. Inferior staining increases from Q1 to Q4, and this increase is correlated with increased total protein values. Quartiles as defined by Schirmer's scores exhibit negative correlations, e.g.,

while the mean Schirmer's score lowers from Q1 to Q4, values for total protein increase. This negative correlation is due to the nature of the Schirmer's scores, where higher values (Q1) indicate a healthy tear production.

[000115] Table 6 shows quartile analysis for TFBUT, inferior staining and Schirmer's Test. T-test values, where significant (< 0.05), are highlighted in **bold**.

Table 6:

TFBUT				Mean values
	Mean	n	% of eyes	Total Protein
Q1	12.80	48	24.6%	1.292
Q4	2.34	48	24.6%	1.318
Q4-Q1	-10.46			0.026
Q1 vs. Q4, t test				<i>0.800</i>
Inferior Staining				
	Mean	n	% of eyes	Total Protein
Q1	0.42	52	27.5%	1.149
Q4	2.25	59	31.2%	1.420
Q4-Q1	1.83			0.271
Q1 vs. Q4, t test				<i>0.006</i>
Schirmer's Test				
	Mean	n	% of eyes	Total Protein
Q1	32.44	52	26.40%	1.111
Q4	5.21	52	26.40%	1.505
Q4-Q1	-27.23			0.394
Q1 vs. Q4, t test				<i>>0.001</i>

[000116] A second round of quartile analysis used the same approach to determine whether quartiles defined by tear constituent values show similar correlations with other metrics of the signs and symptoms of dry eye disease. These data are shown in Table 7.

[000117] Table 7. Quartile analysis for total protein. T-test values, where significant (< 0.05), are highlighted **in bold**.

Protein				Mean values					
	Means	N	% of eyes	TFBUT	OSDI	Ora Calibra Ocular Discomfort	Inferior	Corneal Sum	Schirmer's
Q1	0.85	75	37.88%	6.61	12.17	1.08	1.18	2.68	21.08
Q4	1.79	71	35.86%	5.58	14.55	1.27	1.59	3.53	13.39
Q1-Q4				1.03	-2.38	-0.19	-0.41	-0.85	7.69
Q1 vs. Q4, t-test				<i>0.173</i>	<i>0.366</i>	<i>0.385</i>	0.002	0.006	>0.001

[000118] The quartiles associated with total protein measured displayed a significant difference for corneal staining measures, with inferior and total corneal staining showing a positive correlation with increases in protein levels from Q1 to Q4. Protein quartiles correlate with corneal staining measures.

Discussion

[000119] The current study illustrated the heterogeneity of the two populations of subjects originally enrolled for analysis. Despite their inclusion based upon differential criteria for symptomology, TFBUT and corneal staining, no significant differences between the two populations were identified in the tear constituent analysis.

[000120] In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis. In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis, and comparing tear constituent analysis to tests such as, but not limited to, Schirmer's test, TFBUT, etc., so as to obtain information to treat a patient diagnosed with dry eye disease.

[000121] The quartile analyses show the relationships between traditional metrics and the tested parameters which are part of the tear constituents. An exception to this is TFBUT, which shows only modest correlations with any of the measured tear constituents. In contrast, corneal

staining measures (such as inferior staining, Table 6) are well-correlated with changes in the tested parameters. This is consistent with a diagnosis of evaporative dry eye, where a reduction in aqueous content of the tears would yield apparent increases in the concentrations of all tear constituents. Alternatively, the increases in tear constituent concentration(s) can result from an inflammatory response to ocular surface distress that initiates a shift in the ratio of serious to mucus lacrimal secretions.

[000122] In some embodiments, the method of the present invention includes the use of at least one diagnostic test. In some embodiments, in performing such a comparison of tear constituents in healthy and dry eye subjects, a multiplicative effect is obtained. In some embodiments, a kit is used to provide an assessment between severe patients and healthy subjects.

Example 2: Measurement of Mucin in Tear Samples

[000123] The levels of a prominent tear constituent was examined in healthy subjects and in subjects who met one or more criteria of mild to moderate dry eye. The following experiments illustrate a comparison between benchmark testing for assessment of dry eye with a quantitative measure of a tear constituent. Examples of the tests used to quantitatively measure at least one tear constituent are corneal staining, Schirmer's tests, TFBUT, and provided symptom assessment including the OSDI questionnaire and the Ora-Calibra™ ocular discomfort score. The OSDI is a 12 question assessment that has become a standard for dry eye symptomology. The Ora-Calibra assessments for discomfort also provide a measurement of symptomology by allowing a patient to answer questions, where the number of questions is reduced compared to the OSDI. Samples of tears were collected using capillary tubes and then underwent analysis for the tear constituent. The tear constituent measured was mucin.

Tear constituent assay and measurement methodology:

[000124] Rapid test strips (tear analyzing strips) and reagents were used to measure mucin levels using a semi-quantitative technique; where the semi-quantitative technique followed a fixed running time for each type of assay, strips were scanned with HP's scanner model scanjet 200. The resulting scanned figure was optimized using Function Lighten / Darken: Highlights – (-) 50; Shadows – (-) 69; Midtones – (-) 50; Gamma- 1.7 followed by recording of signal intensity (as shown in Figure 1). Determination of the tear constituent was conducted using semi-quantitative estimation of the intensity test lines compared to intensity of a printer picture of color intensities.

Experimental Design:Subject Population

[000125] Subjects for the study included anyone over the age of 18 years who met the inclusion and exclusion criteria listed in the following tables. The study population included two groups of subjects (Group A, as shown in Table 8, and Group B, shown in Table 9) with approximately equal numbers of each (~ 100 subjects per group):

Table 8: Group A – Healthy Eyes

<i>Healthy Subjects, Inclusion Criteria</i>	
1.	Subject must be 18 years of age and may be of any race and either gender;
2.	The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3.	Subject agrees for samples to be taken from both eyes;
4.	Subject must be willing to follow the study procedures and visit schedule;
5.	Subject must report <2 in all symptoms (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
6.	Subject has at least one of the following in the collection eye(s): <2 in all regions of the cornea (Ora Calibra™ Scale) during visit; - TFBUT > 10 seconds during visit.
<i>Healthy Subjects, Exclusion Criteria</i>	
1.	Subject complained of dry eye or any other acute ocular disease ;
2.	Subject is currently suffering from active inflammation or infection;
3.	Subject used artificial tear drops in the past 2 months;
4.	Subject currently treated medically for a chronic eye syndrome such as glaucoma, allergy or conjunctivitis;
5.	Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or which would present a special risk to the subject;
6.	Subject reports currently being pregnant or nursing;
7.	Use of investigational study drug or study device within 30 days of enrollment.

Table 9: Group B – Suspected Dry Eye

<i>Suspected Dry Eye, Inclusion Criteria</i>
1. Subject must be 18 years of age and may be of any race and either gender;
2. The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3. Subject used or had the desire to use artificial tears in the last 30 days;
4. Subject reports ≥ 2 in at least one symptom (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
5. Subject demonstrates both of the following in the collection eye(s): a. ≥ 2 in at least one region (Ora Calibra™ scale) b. TFBUT < 10 seconds during visit;
6. Subject agrees for samples to be taken from both eyes;
7. Subject must be willing to follow the study procedures and visit schedule.
<i>Suspected Dry Eye, Exclusion Criteria</i>
1. Subject is using contact lenses on a regular basis;
2. Subject is currently suffering from active inflammation or infection;
3. Subject has used Restasis® in the last 30 days;
4. Subject used artificial tear drops in the past hour;
5. Subject is being medically treated for glaucoma;
6. Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or would present a special risk to the subject;
7. Subject reports currently being pregnant or nursing;
8. Subject has participated in any other clinical trial within 30 days of enrollment.

Experimental Design and Methods

[000126] An exemplary embodiment of the method of the present invention was a prospective, single-center, single-visit, parallel-group, data and tear collection study, consisting of approximately 200 subjects. There was one scheduled study visit where subjects were screened; those who met the eligibility criteria were enrolled in the study.

Tear Sample Collection

[000127] The procedure for tear sample collection was as follows:

1. The slit-lamp was set at a low intensity beam.
2. The lower lid of the eye was retracted and a glass capillary tube was placed on the temporal aspect touching the tear surface.
3. The tear surface was contacted and allowed for the collection of between 6 to 25 microliters of tear solution.
4. Once a sufficient volume (e.g., but not limited to, 6 to 25 microliters) was collected, the contents of the glass capillary was withdrawn and emptied into a vial. If tear volume was below 6 microliters, a second sample was drawn from the other eye into another clean vial.
5. The vials were marked with a designated subject label provided by the sponsor.
6. The vials were stored at a temperature of 2°C-8°C. Tear samples were transferred to the sponsor laboratories for initial preparation up to 48 hours from collection before further analysis for levels of mucin.
7. The tear volume was measured within the 48 hours from sampling using pipette of small volume. Two sample volumes of Phosphate buffer saline (PBS X 1) were added to

collected sample followed by a short vortex (20 Sec.) for mixing. Diluted samples were placed back for storage in a temperature of 2°C-8°C.

Mucin Assay

[000128] The assay allows for detection of the mucin in human tears by detecting sugar groups of mucin (a glycoprotein, i.e. containing at least one sugar moiety) using a Lateral Flow immunochromatographic assay. First, a diluted tear sample was placed on the sample pad. Then, additional drops of washing solution were placed on the sample pad to allow the tear sample to migrate and wet the conjugate pad. The conjugate pad contained a first lectin (e.g., Jacalin) conjugated to gold particles through biotin-Avidin interaction. The conjugated lectin bound the mucin from the tear sample and migrated through the nitrocellulose membrane towards the wick. When the gold conjugate/mucin complexes reached the test zone, the gold conjugate/mucin react with a second lectin (wheat germ agglutinin (“WGA”)) fixed to the membrane surface (i.e., at the test line). The accumulation of the gold conjugate/mucin bound to the test line form a pinkish red visible line. An excess amount of complex then migrated to a second zone containing biotin BSA and bound a streptavidin gold conjugate, which formed a second line (a control line). The control line indicated test validity. A residual amount of conjugate and tear sample migrated from the nitrocellulose membrane into the wick pad.

[000129] The test strip was produced as follows: 1mg/mL (0.75-1.5mg/mL) WGA was impregnated onto a chromatographic membrane of nitrocellulose (e.g., Whatman’s paper, FF120). Impregnation is in the shape of a 1mm wide line. The lectin solution additionally contains the following: (1) buffer, e.g., phosphate buffered saline at pH 7.4 or Tris, HEPES, Borax, or MES buffer with pH value ranging from 6.5 – 9.0; (2) 2% trehalose or sucrose, ranging from 1% - 4% concentration; (3) 1-4% ethanol (e.g., but not limited to, 1%, 2%, 3%, 4%

ethanol). The WGA impregnated nitrocellulose was dried at 50 degrees C for 10 minutes to bind the protein to the nitrocellulose. Binding of the WGA to nitrocellulose can also occur between 37 – 60 degrees C for 5 to 24 hours, where a higher temperature would allow for a shorter incubation time. The biotin was bound to Jacalin by conjugating biotin to Jacalin at a ratio of, e.g., but not limited to, 11:1, 22:1, or 33:1. Biotin-Jacalin was bound to streptavidin-gold conjugate at a ratio of 5ug/ml biotin-Jacalin and between OD0.5/mL – OD2.0/mL, e.g., but not limited to, OD1/mL, gold-streptavidin. The reaction complex can also include wash reagent, which clears excess gold conjugates from the nitrocellulose membrane. The wash reagent can contain the following: (1) PBS x 1 at pH 7.4 (can range from pH 7.0 – 9.0); (2) 1% fatty-acid free bovine serum albumin (can range from 0.5% - 3.0%); (3) 0.1% Tween 20 (can range from 0.05% - 2.0%); or any combination thereof. Additionally, 0.05% sodium dodecylsulfate may be added to the wash reagent at a concentration from 0.01% - 1.0%. Regarding Figure 1, the line intensity of 1 is formed when mucin is measured at 12.5µg/mL.

Tear Film Break Up Time Test

[000130] The procedure for TFBUT included:

1. A medical professional instilled 5 μ L of 2% preservative-free sodium fluorescein solution into the inferior conjunctival cul-de-sac of each eye. To thoroughly mix the fluorescein with the tear film, the subject was instructed to blink several times. In order to achieve maximum fluorescence, the medical professional waited approximately 30 seconds after instillation before evaluating TFBUT.
2. With the aid of a slit lamp, the medical professional monitored the integrity of the tear film, noting the time it takes to form micelles from the time that the eye was opened. TFBUT was measured in seconds using a stopwatch and a digital image recording system for the right eye followed by the left eye. A Wratten #12 yellow filter was used to enhance the ability to grade TFBUT.
3. For each eye, two measurements were taken and averaged unless the two measurements were > 2 seconds apart and were each < 10 seconds, in which case, a third measurement was taken and the two closest of the three was averaged.

Corneal Fluorescein Staining:

[000131] The procedure for corneal fluorescein included:

1. In order to achieve maximum fluorescence, the medical professional waited approximately 3-5 minutes after instillation before evaluating fluorescein staining. A Wratten #12 yellow filter was used to enhance the ability to grade fluorescein staining.
2. The inter-palpebral was graded and recorded, and the conjunctiva and cornea epithelial were stained by use of a 5 point scale. The upper eyelid was lifted slightly to

grade the whole corneal surface. Regarding conjunctiva, temporal zone grading was performed when the subject looks nasally; grading nasally by looking temporally.

3. The conjunctival and corneal staining was graded and recorded using the Ora Calibra™ Corneal and Conjunctival Staining Scale.

Unanesthetized Schirmer's Test

[000132] The Schirmer Tear Test was performed according to the following procedure:

1. Using a sterile Tear Flo Schirmer's test strip (e.g., obtained from, but not limited to, Rose Enterprises), a bend in the strip was made in line with the notch in the strip.
2. The subject will be instructed to gaze up and in.
3. The Schirmer's test strip was placed in the lower temporal lid margin of each eye such that the strip fits tightly. Subjects were instructed to close their eyes.
4. After 5 minutes have elapsed, the Schirmer's strip was removed. The length of the moistened area was recorded (mm) for each eye.

Ora Calibra™ Ocular Discomfort Scale

[000133] In an exemplary embodiment, ocular discomfort scores were subjectively graded by the subjects according to the following scale, rating each eye separately. The scale used is shown below and ranges from 0-4:

0 = no discomfort

1 = intermittent awareness

2 = constant awareness

3 = intermittent discomfort

4 = constant discomfort

Ora Calibra™ Ocular Discomfort & 4-Symptom Questionnaire

[000134] Subjects rated the severity of each of the following symptoms, with regards to how both their eyes felt, in general – overall ocular discomfort, burning, dryness, grittiness and stinging according to the following 6-point (0 to 5) scale where 0 = none and 5 = worst.

0	1	2	3	4	5
(None)					(Most)

[000135] Standards of professional care to protect the ocular safety of subjects were followed with regard to study regimen adherence. Subjects who met entry criteria provided demographic information, medical and ocular history and artificial tears use if appropriate. Clinical staff confirmed that subjects did not use artificial tears in the hour prior to the study, then guided subjects through the following procedures:

- (1) Subjects completed the OSDI© questionnaire and Ora Calibra™ Ocular Discomfort & 4-symptom Questionnaire.
- (2) Subjects and staff reviewed source documents to confirm that subject met all inclusion/exclusion criteria based on current medications and medical history.
- (3) Clinical staff collected 6-25 microliters of tears using a capillary from the right eye of the subject. Staff labeled the collection vial with the subject screening number and emptied the capillary contents into the vial.
- (4) In the cases where tear volume collected from the right eye was below 6 microliters, a sample was drawn from the left eye and the capillary was emptied into another clean vial marked with same subject screening number.

- (5) Clinical staff performed tear film break up time test on collection eye(s).
- (6) Clinical staff performed Corneal Fluorescein Staining and examined the ocular surface of the collection eye(s).
- (7) If 6 or greater microliters were collected from the right eye, but the subject did not meet Tear Film Break Up Time or Fluorescein Staining inclusion criteria in the right eye, steps 3-6 were repeated in left eye.
- (8) Clinical staff performed un-anesthetized Schirmer's Test on collection eye(s).
- (9) Clinical staff reviewed results to determine if patient met all inclusion/exclusion criteria based on data collected according to items 3-8.
- (10) Patients who met all criteria were assigned a subject study number and categorized on label based on diagnosis of healthy or suspected dry eye patient.
- (11) Adverse events, if applicable, were documented.

[000136] Samples were handled and tested using the following parameters:

- (1) Volume of collected tears was measured using a micropipette. Twice the measured volume was added with Phosphate Buffer Saline (PBS) for a final dilution of 1:3.
- (2) Diluted tears were further diluted serially to the following dilutions: 1:50, 1:100 and 1:200 with PBS.
- (3) Two microliters of diluted sample were mixed with 18 microliters of gold conjugate mix in a microtube. The relevant test strip was dipped in that mix for 4 minutes.
- (4) Additional 25 microliters of wash solution were added to the tube for excess dye clearance from reaction zone.
- (5) After 6 minutes developed test strips were gently blot against tissue paper and scanned with a desk scanner.

(6) Test intensity was quantified according to the intensity scale presented in Figure 1.

Power Analysis:

[000137] Table 10 presents power for selected sample sizes.

Table 10:

Performance Goal	Sample Proportion	N	Power
0.55	0.70	95	0.82
	0.72	75	0.82
	0.75	50	0.84
0.60	0.70	100	0.55
	0.72	100	0.71
	0.75	90	0.84
0.65	0.70	100	0.16
	0.72	100	0.29
	0.75	100	0.55

[000138] The power was estimated using Exact Binomial method, where two co-primary endpoints (sensitivity and specificity) were taken into account, and where “N” represents the number of positive only (or negative only) cases. Thus, the total sample size was doubled.

[000139] Table 11 illustrates a “Precision” parameter, which is defined as a half-length of confidence interval (CI). The CI is an interval estimate of a population parameter. The CI is an observed interval (i.e. it is calculated from the observations), in principle different from sample to sample, that frequently includes the parameter of interest if the experiment is repeated.

Table 11: Confidence Interval Precision

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
80	48	60.0%	48.4%	70.8%	11.2%
	52	65.0%	53.5%	75.4%	11.0%
	56	70.0%	58.7%	79.8%	10.6%
	60	75.0%	64.0%	84.1%	10.1%

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
	64	80.0%	69.5%	88.2%	9.4%
	68	85.0%	75.2%	92.1%	8.5%
	72	90.0%	81.2%	95.6%	7.2%
90	54	60.0%	49.1%	70.2%	10.6%
	59	65.6%	54.7%	75.3%	10.3%
	63	70.0%	59.4%	79.3%	10.0%
	68	75.6%	65.3%	84.1%	9.4%
	72	80.0%	70.2%	87.7%	8.8%
	77	85.6%	76.5%	92.1%	7.8%
	81	90.0%	81.8%	95.4%	6.8%
100	60	60.0%	49.7%	69.7%	10.0%
	65	65.0%	54.8%	74.3%	9.8%
	70	70.0%	60.0%	78.8%	9.4%
	75	75.0%	65.3%	83.2%	9.0%
	80	80.0%	70.8%	87.4%	8.3%
	85	85.0%	76.4%	91.4%	7.5%
	90	90.0%	82.3%	95.1%	6.4%

Results and Analysis

[000140] The primary outcome of the study was the comparison of benchmark tests for dry eye such as TFBUT, Corneal staining, Schirmer's test, and OSDI questionnaires with results from a test of tear film constituents (e.g., mucin).

[000141] All collected samples obtained from patients' eyes which met the entry criteria were included in the analyses. The goal of the study was to develop an assessment tool to compare benchmark tests for dry eye with a kit that tests the tear film compound, mucin. Data was distributed from lowest to highest values and compared with other parameters to identify positive and negative correlations.

[000142] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 –

12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by Schirmer's test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by Schirmer's test.

[000143] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by a corneal staining test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by a corneal staining test.

[000144] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by OSDI. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., mucin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by OSDI.

[000145] Sample size in this pilot study (200 total eyes, 100 per group) was not based on any power analysis, but was based on an approximation of the number of eyes sufficient to build a model for a distinguishing between healthy and suspected dry eye tears and evaluation of benchmark standard testing with the different tested parameters.

[000146] Adverse Events (AEs) included any events reported over the course of the tear collection and ocular surface assessment procedures. This clinical study involved TFBUT, corneal staining and the collection of tears for the constituent analysis. During these tests the

participant may have felt a foreign body sensation. During the tear collection there may have been cases of direct contact with the eye due to movement, resulting in corneal abrasion, or eye redness. Any such events were noted and graded as follows:

- Mild: Sign or symptom, usually transient, requiring no special treatment, generally not interfering with usual activities.
- Moderate: Sign or symptom, which may be ameliorated by simple therapeutic measures; may interfere with usual activity.
- Severe: Sign or symptom that are intense or debilitating and that interfere with usual activities. Recovery was usually aided by therapeutic measures.

Results

[000147] A total of 198 subjects completed the study, including 126 women and 72 men. The breakdown of subjects according to entry criteria A or B is outlined in Table 12 below. Those who met entry criteria were not matched for age or gender in this study.

Table 12:

	Men	Women	mean age
Group A Healthy	41	59	45.5
Group B Suspected DE	31	67	58.6

Analysis

[000148] Subjects enrolled in each study group met the entry criteria of either healthy or suspected dry eye. The only demographic criteria that showed a significant difference between the two groups was age; preliminary analysis showed no significant difference in any tear metrics between the two groups. In addition, both groups displayed a range of values for the benchmark testing parameters. Based upon this observation, all subjects were pooled into a single group and analyzed using population quartiles with an assumption that the population sampled represented a continuum of dry eye severity. Using this concept, measurements for each

of the benchmark tests were ranked, and mean values for each of 4 quartiles were compared to measures for the tear diagnostics.

Quartile Analysis

[000149] The quartile analysis for TFBUT, inferior staining, and Schirmer's tests are summarized in Table 13. The focus of this approach was on the extremes, quartiles 1 and 4, as these represent those patients with the largest differences for each metric. In all three measures, Q1 was the quartile with values expected for normal patients and Q4 was the quartile with values associated with dry eye disease. For example, those in Q1 have a mean TFBUT of 12.80 seconds and so would be considered normal while those in Q4 have a mean TFBUT of 2.34 seconds, consistent with a diagnosis of moderate dry eye disease. When the mean values for tested parameters in each of the TFBUT-defined quartiles and/or corneal staining were compared, associations between the break-up time metric and tear constituent dynamics emerged. The decrease in TFBUT between Q1 and Q4 is accompanied by a decrease in mucin. Quartiles defined by Schirmer's scores show negative correlations, e.g., while the mean Schirmer's score is reduced from Q1 to Q4, the amount of mucin increases. The mucin-defined quartiles show significant correlation with corneal staining scores, and also exhibit a correlation with symptom scores OSDI and Ora Calibra Ocular Discomfort scores. Increased mucin values are correlate with greater symptom scores, stronger corneal staining scores, and reduced Schirmer's scores.

[000150] Table 13 shows quartile analysis for TFBUT, inferior staining and Schirmer's Test. T-test values, where significant (< 0.05), are highlighted **in bold**.

Table 13:

TFBUT				Mean values
	Mean	n	% of eyes	Mucin
Q1	12.80	48	24.6%	0.579
Q4	2.34	48	24.6%	0.443
Q4-Q1	-10.46			-0.135
Q1 vs. Q4, t test				0.103
Inferior Staining				
	Mean	n	% of eyes	Mucin
Q1	0.42	52	27.5%	0.351
Q4	2.25	59	31.2%	0.646
Q4-Q1	1.83			0.295
Q1 vs. Q4, t test				0.003
Schirmer's Test				
	Mean	n	% of eyes	Mucin
Q1	32.44	52	26.40%	0.466
Q4	5.21	52	26.40%	0.624
Q4-Q1	-27.23			0.158
Q1 vs. Q4, t test				0.047

[000151] A second round of quartile analysis used the same approach to determine whether quartiles defined by tear constituent values show similar correlations with other metrics of the signs and symptoms of dry eye disease. These data are shown in Table 14.

Table 14. Quartile analysis for mucin. T-test values, where significant (< 0.05), are highlighted in bold.

Mucins				Mean Values					
	Means	N	% of eyes	TFBUT	OSDI	Ora Calibra Ocular Discomfort	Inferior	Corneal Sum	Schirmer's
Q1	0.07	53	26.77%	5.30	11.26	0.98	1.08	2.45	19.68
Q4	0.93	78	39.39%	6.55*	17.59	1.44	1.37	3.06	15.09
Q1-Q4				-1.25	-6.33	-0.45	-0.29	-0.62	4.59
Q1 vs. Q4, t-test				0.106	0.044	0.018	0.062	0.011	0.015
Q1 vs. Q4, t-test				0.173	0.366	0.385	0.002	0.006	>0.001

[000152] The quartiles associated with lacrimal gland protein mucin displayed a significant difference for corneal staining measures, with inferior and total corneal staining showing a positive correlation with increases in protein levels from Q1 to Q4.

Discussion

[000153] The current study illustrated the heterogeneity of the two populations of subjects originally enrolled for analysis. Despite their inclusion based upon differential criteria for symptomology, TFBUT and corneal staining, no significant differences between the two populations were identified in the tear constituent analysis.

[000154] In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis. In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis, and comparing tear constituent analysis to tests such as, but not limited to, Schirmer's test, TFBUT, etc., so as to obtain information to treat a patient diagnosed with dry eye disease.

[000155] The quartile analyses show the relationships between traditional metrics and the tested parameters which are part of the tear constituents. An exception to this is TFBUT, which shows only modest correlations with any of the measured tear constituents. In contrast, corneal

staining measures (such as inferior staining, Table 13) are well-correlated with changes in the tested parameters. This is consistent with a diagnosis of evaporative dry eye, where a reduction in aqueous content of the tears would yield apparent increases in the concentrations of all tear constituents. Alternatively, the increases in tear constituent concentration(s) can result from an inflammatory response to ocular surface distress that initiates a shift in the ratio of serious to mucus lacrimal secretions.

[000156] In some embodiments, the method of the present invention includes the use of at least one diagnostic test. In some embodiments, in performing such a comparison of tear constituents in healthy and dry eye subjects, a multiplicative effect is obtained. In some embodiments, a kit is used to provide an assessment between severe patients and healthy subjects.

Example 3: Measurement of Lactoferrin in Tear Samples

[000157] The levels of a prominent tear constituent was examined in healthy subjects and in subjects who met one or more criteria of mild to moderate dry eye. The following experiments illustrate a comparison between benchmark testing for assessment of dry eye with a quantitative measure of a tear constituent. Examples of the tests used to quantitatively measure at least one tear constituent are corneal staining, Schirmer's tests, TFBUT, and provided symptom assessment including the OSDI questionnaire and the Ora-Calibra™ ocular discomfort score. The OSDI is a 12 question assessment that has become a standard for dry eye symptomology. The Ora-Calibra assessments for discomfort also provide a measurement of symptomology by allowing a patient to answer questions, where the number of questions is reduced compared to the OSDI. Samples of tears were collected using capillary tubes and then underwent analysis for the tear constituent. The tear constituent measured was lactoferrin.

Tear constituent assay and measurement methodology:

[000158] Rapid test strips (tear analyzing strips) and reagents were used to measure lactoferrin levels using a semi-quantitative technique; where the semi-quantitative technique followed a fixed running time for each type of assay, strips were scanned with HP's scanner model scanjet 200. The scanned figure was optimized using Function Lighten / Darken: Highlights – (-) 50; Shadows – (-) 69; Midtones – (-) 50; Gamma- 1.7 followed by recording of signal intensity (shown in Figure 1). Determination of the tear constituent was conducted using semi-quantitative estimation of the intensity test lines compared to intensity of a series of control lines.

Experimental Design:Subject Population

[000159] Subjects for the study included anyone over the age of 18 years who met the inclusion and exclusion criteria listed in the following tables. The study population included two groups of subjects (Group A, as shown in Table 15, and Group B, shown in Table 16) with approximately equal numbers of each (~ 100 subjects per group):

Table 15: Group A – Healthy Eyes

<i>Healthy Subjects, Inclusion Criteria</i>	
1.	Subject must be 18 years of age and may be of any race and either gender;
2.	The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3.	Subject agrees for samples to be taken from both eyes;
4.	Subject must be willing to follow the study procedures and visit schedule;
5.	Subject must report <2 in all symptoms (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
6.	Subject has at least one of the following in the collection eye(s): <2 in all regions of the cornea (Ora Calibra™ Scale) during visit; - TFBUT > 10 seconds during visit.
<i>Healthy Subjects, Exclusion Criteria</i>	
1.	Subject complained of dry eye or any other acute ocular disease ;
2.	Subject is currently suffering from active inflammation or infection;
3.	Subject used artificial tear drops in the past 2 months;
4.	Subject currently treated medically for a chronic eye syndrome such as glaucoma, allergy or conjunctivitis;
5.	Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or which would present a special risk to the subject;
6.	Subject reports currently being pregnant or nursing;
7.	Use of investigational study drug or study device within 30 days of enrollment.

Table 16: Group B – Suspected Dry Eye

<i>Suspected Dry Eye, Inclusion Criteria</i>
1. Subject must be 18 years of age and may be of any race and either gender;
2. The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3. Subject used or had the desire to use artificial tears in the last 30 days;
4. Subject reports ≥ 2 in at least one symptom (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
5. Subject demonstrates both of the following in the collection eye(s): a. ≥ 2 in at least one region (Ora Calibra™ scale) b. TFBUT < 10 seconds during visit;
6. Subject agrees for samples to be taken from both eyes;
7. Subject must be willing to follow the study procedures and visit schedule.
<i>Suspected Dry Eye, Exclusion Criteria</i>
1. Subject is using contact lenses on a regular basis;
2. Subject is currently suffering from active inflammation or infection;
3. Subject has used Restasis® in the last 30 days;
4. Subject used artificial tear drops in the past hour;
5. Subject is being medically treated for glaucoma;
6. Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or would present a special risk to the subject;
7. Subject reports currently being pregnant or nursing;
8. Subject has participated in any other clinical trial within 30 days of enrollment.

Experimental Design and Methods

[000160] An exemplary embodiment of the method of the present invention was a prospective, single-center, single-visit, parallel-group, data and tear collection study, consisting of approximately 200 subjects. There was one scheduled study visit where subjects were screened; those who met the eligibility criteria were enrolled in the study.

Tear Sample Collection

[000161] The procedure for tear sample collection was as follows:

1. The slit-lamp was set at a low intensity beam.
2. The lower lid of the eye was retracted and a glass capillary tube was placed on the temporal aspect touching the tear surface.
3. The tear surface was contacted and allowed for the collection of between 6-25 microliters of tear solution.
4. Once a sufficient volume (e.g., but not limited to, 6-25 microliters) was collected, the contents of the glass capillary was withdrawn and emptied into a vial. If tear volume was below 6 microliters, a second sample was drawn from the other eye into another clean vial.
5. The vials were marked with a designated subject label provided by the sponsor.
6. The vials were stored at a temperature of 2°C-8°C. Tear samples were transferred to the sponsor laboratories for initial preparation up to 48 hours from collection before further analysis for levels of lactoferrin.
7. The tear volume was measured within the 48 hours from sampling using pipette of small volume. Two sample volumes of Phosphate buffer saline (PBS X 1) were added to

collected sample followed by a short vortex (20 Sec.) for mixing. Diluted samples were placed back for storage in a temperature of 2°C-8°C.

Lactoferrin Assay

[000162] The assay allows for direct detection of the lactoferrin in human tears using specific detection of sugar groups of lactoferrin (i.e. a glycoprotein,) using a Lateral Flow immunochromatographic assay. First, 20 microliters of tear sample diluted 1:2000 was placed on the sample pad. Then, additional 40 µL of washing solution were placed on the sample pad to allow the tear sample to migrate and wet the conjugate pad. The conjugate pad contained a first lectin (e.g., pisum sativum agglutinin (“PSA”)) conjugated to streptavidin conjugated to gold particles [manufactured by Arista Biologicals Inc. 1101 Hamilton Street, Allentown, PA 18101] through biotin avidin interaction. The conjugated lectin bound the lactoferrin from the tear sample and migrated through the nitrocellulose membrane towards the wick. When the gold conjugate/lactoferrin complexes reached the test zone, the gold conjugate/lactoferrin bound to the second lectin (e.g., lens culinaris agglutinin (“LCA”)) fixated to the membrane surface (i.e., at the test line). The accumulation of the gold conjugate/lactoferrin bound to the test line form a pinkish red visible line. An excess amount of complex then migrated to a second zone containing biotin BSA that bounds the streptavidin gold conjugate. A second line is formed (a control line). The control line indicated test validity. A residual amount of conjugate and tear sample migrated from the nitrocellulose membrane into the wick pad.

[000163] The test strip was produced as follows: 1mg/mL (0.75-1.5mg/mL) LCA was impregnated onto a chromatographic membrane of nitrocellulose (e.g., Whatman’s nitrocellulose membrane, FF120 but can also be mdi CNPH-N-5560 membrane). LCA was impregnated on the test strip in the shape of a 1mm wide line. The LCA solution additionally contains the following:

(1) buffer, e.g., phosphate buffered saline at pH 7.4 or Tris, HEPES, Borax, or MES buffer with pH value ranging from 6.5 – 9.0; (2) 2% trehalose or sucrose, ranging from 1% - 4% concentration; (3) 1-4% ethanol (e.g., but not limited to, 1%, 2%, 3%, 4% ethanol). The LCA impregnated nitrocellulose was dried at 50 degrees C for 10 minutes to bind the protein to the nitrocellulose. Binding of the LCA to nitrocellulose can also occur between 37 – 60 degrees C for 5 to 24 hours, where a higher temperature would allow for a shorter incubation time. The biotin was bound to PSA by conjugating biotin to PSA at a ratio of, e.g., but not limited to, 11:1, 22:1, or 33:1. Biotin-PSA was bond to streptavidin-gold conjugate at a ratio of 5ug/ml biotin-PSA (but can range from 1ug/ml to 7ug/ml of concentration) and between OD0.5/mL – OD2.0/mL, e.g., but not limited to, OD1/mL, gold-streptavidin. The reaction complex can also include wash reagent, which clears excess gold conjugates from the nitrocellulose membrane. The wash reagent can contain the following: (1) PBS x 1 at pH 7.4 (can range from pH 7.0 – 9.0); (2) 1% fatty-acid free bovine serum albumin (can range from 0.5% - 3.0%); (3) 0.1% Tween 20 (can range from 0.05% - 2.0%); (4) 0.05% sodium dodecylsulfate (can range from 0.01% - 1%), or any combination thereof. Regarding Figure 1, the line intensity of 1 is formed when lactoferrin is measured at 50ug/mL (i.e., showing equivalence between line intensity and lactoferrin concentration).

Tear Film Break Up Time Test

[000164] The procedure for TFBUT included:

1. A medical professional instilled 5 μ L of 2% preservative-free sodium fluorescein solution into the inferior conjunctival cul-de-sac of each eye. To thoroughly mix the fluorescein with the tear film, the subject was instructed to blink several times. In order to achieve maximum fluorescence, the medical professional waited approximately 30 seconds after instillation before evaluating TFBUT.
2. With the aid of a slit lamp, the medical professional monitored the integrity of the tear film, noting the time it takes to form micelles from the time that the eye was opened. TFBUT was measured in seconds using a stopwatch and a digital image recording system for the right eye followed by the left eye. A Wratten #12 yellow filter was used to enhance the ability to grade TFBUT.
3. For each eye, two measurements were taken and averaged unless the two measurements were greater than 2 seconds apart and were each less than 10 seconds, in which case, a third measurement was taken and the two closest of the three was averaged.

Corneal Fluorescein Staining:

[000165] The procedure for corneal fluorescein included:

1. In order to achieve maximum fluorescence, the medical professional waited approximately 3-5 minutes after instillation before evaluating fluorescein staining. A Wratten #12 yellow filter was used to enhance the ability to grade fluorescein staining.
2. The inter-palpebral was graded and recorded, and the conjunctiva and cornea epithelial were stained by use of a 5 point scale (e.g., pictures of scanned strips/panel which had line intensity representing one degree of the intensities scale). The upper

eyelid was lifted slightly to grade the whole corneal surface. Regarding conjunctiva, temporal zone grading was performed when the subject looks nasally; grading nasally by looking temporally.

3. The conjunctival and corneal staining was graded and recorded using the Ora Calibra™ Corneal and Conjunctival Staining Scale.

Unanesthetized Schirmer's Test

[000166] The Schirmer Tear Test was performed according to the following procedure:

1. Using a sterile Tear Flo Schirmer's test strip (e.g., obtained from, but not limited to, Rose Enterprises), a bend in the strip was made in line with the notch in the strip.
2. The subject will be instructed to gaze up and in.
3. The Schirmer's test strip was placed in the lower temporal lid margin of each eye such that the strip fits tightly. Subjects were instructed to close their eyes.
4. After 5 minutes have elapsed, the Schirmer's strip was removed. The length of the moistened area was recorded (mm) for each eye.

Ora Calibra™ Ocular Discomfort Scale

[000167] In an exemplary embodiment, ocular discomfort scores were subjectively graded by the subjects according to the following scale, rating each eye separately. The scale used is shown below and ranges from 0-4:

0 = no discomfort

1 = intermittent awareness

2 = constant awareness

3 = intermittent discomfort

4 = constant discomfort

Ora Calibra™ Ocular Discomfort & 4-Symptom Questionnaire

[000168] Subjects rated the severity of each of the following symptoms, with regards to how both their eyes felt, in general – overall ocular discomfort, burning, dryness, grittiness and stinging according to the following 6-point (0 to 5) scale where 0 = none and 5 = most.

0	1	2	3	4	5
(None)					(Most)

[000169] Standards of professional care to protect the ocular safety of subjects were followed with regard to study regimen adherence. Subjects who met entry criteria provided demographic information, medical and ocular history and artificial tears use if appropriate. Clinical staff confirmed that subjects did not use artificial tears in the hour prior to the study, then guided subjects through the following procedures:

- (1) Subjects completed the OSDI© questionnaire and Ora Calibra™ Ocular Discomfort & 4-symptom Questionnaire.
- (2) Subjects and staff reviewed source documents to confirm that subject met all inclusion/exclusion criteria based on current medications and medical history.
- (3) Clinical staff collected 6-25 microliters of tears using a capillary from the right eye of the subject. Staff labeled the collection vial with the subject screening number and emptied the capillary contents into the vial.
- (4) In the cases where tear volume collected from the right eye was below 6 microliters, a sample was drawn from the left eye and the capillary was emptied into another clean vial marked with same subject screening number.

- (5) Clinical staff performed tear film break up time test on collection eye(s).
- (6) Clinical staff performed Corneal Fluorescein Staining and examined the ocular surface of the collection eye(s).
- (7) If 6 or greater microliters were collected from the right eye, but the subject did not meet Tear Film Break Up Time or Fluorescein Staining inclusion criteria in the right eye, steps 3-6 were repeated in left eye.
- (8) Clinical staff performed un-anesthetized Schirmer's Test on collection eye(s).
- (9) Clinical staff reviewed results to determine if patient met all inclusion/exclusion criteria based on data collected according to items 3-8.
- (10) Patients who met all criteria were assigned a subject study number and categorized on label based on diagnosis of healthy or suspected dry eye patient.
- (11) Adverse events, if applicable, were documented.

[000170] Samples were handled and tested using the following parameters:

- (1) Volume of collected tears was measured using a micropipette. Twice the measured volume was added with Phosphate Buffer Saline (PBS) for a final dilution of 1:3.
- (2) Diluted tears were further diluted serially to the following dilutions: 1:50, 1:100 and 1:200 with PBS.
- (3) Two microliters of diluted sample were mixed with 18 microliters of gold conjugate mix in a microtube. The relevant test strip was dipped in that mix for 4 minutes.
- (4) Additional 25 microliters of wash solution were added to the tube for excess dye clearance from reaction zone.
- (5) After 6 minutes developed test strips were gently blot against tissue paper and scanned with a desk scanner.

(6) Test intensity was quantified according to the intensity scale presented in Figure 1.

Power Analysis:

[000171] Table 17 presents power for selected sample sizes.

Table 17:

Performance Goal	Sample Proportion	N	Power
0.55	0.70	95	0.82
	0.72	75	0.82
	0.75	50	0.84
0.60	0.70	100	0.55
	0.72	100	0.71
	0.75	90	0.84
0.65	0.70	100	0.16
	0.72	100	0.29
	0.75	100	0.55

[000172] The power was estimated using Exact Binomial method, where two co-primary endpoints (sensitivity and specificity) were taken into account, and where “N” represents the number of positive only (or negative only) cases. Thus, the total sample size was doubled.

[000173] Table 18 illustrates a “Precision” parameter, which is defined as a half-length of confidence interval (CI). The CI is an interval estimate of a population parameter. The CI is an observed interval (i.e. it is calculated from the observations), in principle different from sample to sample, that frequently includes the parameter of interest if the experiment is repeated.

Table 18: Confidence Interval Precision

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
80	48	60.0%	48.4%	70.8%	11.2%
	52	65.0%	53.5%	75.4%	11.0%
	56	70.0%	58.7%	79.8%	10.6%
	60	75.0%	64.0%	84.1%	10.1%
	64	80.0%	69.5%	88.2%	9.4%
	68	85.0%	75.2%	92.1%	8.5%
	72	90.0%	81.2%	95.6%	7.2%
	76	95.0%	87.1%	98.8%	6.1%
90	54	60.0%	49.1%	70.2%	10.6%
	59	65.6%	54.7%	75.3%	10.3%
	63	70.0%	59.4%	79.3%	10.0%
	68	75.6%	65.3%	84.1%	9.4%
	72	80.0%	70.2%	87.7%	8.8%
	77	85.6%	76.5%	92.1%	7.8%
	81	90.0%	81.8%	95.4%	6.8%
	86	95.6%	88.1%	98.8%	5.9%
100	60	60.0%	49.7%	69.7%	10.0%
	65	65.0%	54.8%	74.3%	9.8%
	70	70.0%	60.0%	78.8%	9.4%
	75	75.0%	65.3%	83.2%	9.0%
	80	80.0%	70.8%	87.4%	8.3%
	85	85.0%	76.4%	91.4%	7.5%
	90	90.0%	82.3%	95.1%	6.4%

Results and Analysis

[000174] The primary outcome of the study was the comparison of benchmark tests for dry eye such as TFBUT, Corneal staining, Schirmer's test, and OSDI questionnaires with results from a test of tear film constituents (e.g., lactoferrin).

[000175] All collected samples obtained from patients' eyes which met the entry criteria were included in the analyses. The goal of the study was to develop an assessment tool to compare benchmark tests for dry eye with a kit that tests the tear film compound, lactoferrin.

Data was distributed from lowest to highest values and compared with other parameters to identify positive and negative correlations. Figure 1 illustrates the correlation of test line intensity with analyte concentration. In some embodiments, a reduced test line intensity correlates with a test for dry eye (e.g., Schirmer's test, corneal staining, OSDI, etc.).

[000176] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by Schirmer's test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by Schirmer's test.

[000177] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by a corneal staining test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by a corneal staining test.

[000178] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by OSDI. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lactoferrin; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by OSDI.

[000179] Sample size in this pilot study (200 total eyes, 100 per group) was not based on any power analysis, but was based on an approximation of the number of eyes sufficient to build a model for a distinguishing between healthy and suspected dry eye tears and evaluation of benchmark standard testing with the different tested parameters.

[000180] Adverse Events (AEs) included any events reported over the course of the tear collection and ocular surface assessment procedures. This clinical study involved TFBUT, corneal staining and the collection of tears for the constituent analysis. During these tests the participant may have felt a foreign body sensation. During the tear collection there may have been cases of direct contact with the eye due to movement, resulting in corneal abrasion, or eye redness. Any such events were noted and graded as follows:

- Mild: Sign or symptom, usually transient, requiring no special treatment, generally not interfering with usual activities.
- Moderate: Sign or symptom, which may be ameliorated by simple therapeutic measures; may interfere with usual activity.
- Severe: Sign or symptom that are intense or debilitating and that interfere with usual activities. Recovery was usually aided by therapeutic measures.

Results

[000181] A total of 198 subjects completed the study, including 126 women and 72 men. The breakdown of subjects according to entry criteria A or B is outlined in Table 19 below. Those who met entry criteria were not matched for age or gender in this study.

[000182] Table 19:

	Men	Women	mean age
Group A Healthy	41	59	45.5
Group B Suspected DE	31	67	58.6

Analysis

[000183] Subjects enrolled in each study group met the entry criteria of either healthy or suspected dry eye. The only demographic criteria that showed a significant difference between the two groups was age; preliminary analysis showed no significant difference in any tear metrics between the two groups. In addition, both groups displayed a range of values for the benchmark testing parameters. Based upon this observation, all subjects were pooled into a single group and analyzed using population quartiles with an assumption that the population sampled represented a continuum of dry eye severity. Using this concept, measurements for each of the benchmark tests were ranked, and mean values for each of 4 quartiles were compared to measures for the tear diagnostics.

Quartile Analysis

[000184] The quartile analysis for TFBUT, inferior staining, and Schirmer's tests are summarized in Table 20. The focus of this approach was on the extremes, quartiles 1 and 4, as these represent those patients with the largest differences for each metric. In all three measures, Q1 was the quartile with values expected for normal patients and Q4 was the quartile with values associated with dry eye disease. For example, those in Q1 have a mean TFBUT of 12.80 seconds and so would be considered normal while those in Q4 have a mean TFBUT of 2.34 seconds, consistent with a diagnosis of moderate dry eye disease. When the mean values for tested parameters in each of the TFBUT-defined quartiles were compared, associations between the break-up time metric and tear constituent dynamics emerged. The decrease in TFBUT between Q1 and Q4 was accompanied by an increase in lactoferrin. Inferior staining increases from Q1 to Q4, and this increase was significantly correlated with an increase in lactoferrin. Quartiles defined by Schirmer's scores exhibited significant negative correlations: while the

mean Schirmer's score went down from Q1 to Q4, values for lactoferrin increases, and showed a significant difference between Q1 and Q4. This negative correlation was due to the nature of the Schirmer's scores, where higher values (Q1) indicated a healthy tear production.

[000185] Table 20 shows quartile analysis for TFBUT, inferior staining and Schirmer's Test. T-test values, where significant (< 0.05), are highlighted in **bold**.

Table 20:

TFBUT				Mean values
	Mean	n	% of eyes	Lactoferrin
Q1	12.80	48	24.6%	0.979
Q4	2.34	48	24.6%	1.099
Q4-Q1	-10.46			0.120
Q1 vs. Q4, t test				0.037
Inferior Staining				
	Mean	n	% of eyes	Lactoferrin
Q1	0.42	52	27.5%	0.596
Q4	2.25	59	31.2%	0.808
Q4-Q1	1.83			0.212
Q1 vs. Q4, t test				0.003
Schirmer's Test				
	Mean	n	% of eyes	Lactoferrin
Q1	32.44	52	26.40%	0.587
Q4	5.21	52	26.40%	0.803
Q4-Q1	-27.23			0.216
Q1 vs. Q4, t test				>0.001

[000186] A second round of quartile analysis used the same approach to determine whether quartiles defined by tear constituent values show similar correlations with other metrics of the signs and symptoms of dry eye disease. These data are shown in Table 21.

Table 21. Quartile analysis for lactoferrin. T-test values, where significant (< 0.05), are highlighted in **bold**.

Lactoferrin				Mean Values					
	Means	N	% of eyes	TFBUT	OSDI	Ora Calibra Ocular Discomfort	Inferior	Corneal Sum	Schirmer's
Q1	0.42	90	45.45%	6.19	13.66	1.12	1.14	2.66	19.22
Q4	1.05	57	28.79%	5.79	15.35	1.44	1.56	3.41	13.28
Q1-Q4				0.40	-1.69	-0.32	-0.42	-0.76	5.94
Q1 vs. Q4, t-test				<i>0.603</i>	<i>0.544</i>	<i>0.167</i>	0.001	0.010	0.001

[000187] The quartiles associated with lacrimal gland protein lactoferrin displayed a significant difference for corneal staining measures, with inferior and total corneal staining showing a positive correlation with increases in protein levels from Q1 to Q4.

Discussion

[000188] The current study illustrated the heterogeneity of the two populations of subjects originally enrolled for analysis. Despite their inclusion based upon differential criteria for symptomology, TFBUT and corneal staining, no significant differences between the two populations were identified in the tear constituent analysis.

[000189] In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis. In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis, and comparing tear constituent analysis to tests such as, but not limited to, Schirmer's test, TFBUT, etc., so as to obtain information to treat a patient diagnosed with dry eye disease.

[000190] The quartile analyses show the relationships between traditional metrics and the tested parameters which are part of the tear constituents. An exception to this is TFBUT, which shows only modest correlations with any of the measured tear constituents. In contrast, corneal staining measures (such as inferior staining, Table 20) are well-correlated with changes in the

tested parameters. This is consistent with a diagnosis of evaporative dry eye, where a reduction in aqueous content of the tears would yield apparent increases in the concentrations of all tear constituents. Alternatively, the increases in tear constituent concentration(s) can result from an inflammatory response to ocular surface distress that initiates a shift in the ratio of serious to mucus lacrimal secretions. Additionally, greater amounts of lactoferrin correlate with greater staining and lower Schirmer's scores; additionally, lactoferrin shows significant correlation with a lower TFBUT.

Example 4: Measurement of Lysozyme in Tear Samples

[000191] The levels of a prominent tear constituent was examined in healthy subjects and in subjects who met one or more criteria of mild to moderate dry eye. The following experiments illustrate a comparison between benchmark testing for assessment of dry eye with a quantitative measure of a tear constituent. Examples of the tests used to quantitatively measure at least one tear constituent are corneal staining, Schirmer's tests, TFBUT, and provided symptom assessment including the OSDI questionnaire and the Ora-Calibra™ ocular discomfort score. The OSDI is a 12 question assessment that has become a standard for dry eye symptomology. The Ora-Calibra assessments for discomfort also provide a measurement of symptomology by allowing a patient to answer questions, where the number of questions is reduced compared to the OSDI. Samples of tears were collected using capillary tubes and then underwent analysis for the tear constituent. The tear constituent measured was lysozyme.

Tear constituent assay and measurement methodology:

[000192] Rapid test strips/TAS (i.e., tear analyzing strips) and reagents were used to measure lysozyme levels using a semi-quantitative technique; where the semi-quantitative technique followed a fixed running time for each type of assay, strips were scanned with HP's

scanner model scanjet 200. The resulting scanned figure was optimized using Function Lighten / Darken: Highlights – (-) 50; Shadows – (-) 69; Midtones – (-) 50; Gamma- 1.7 followed by recording of signal intensity (as shown in Figure 1). Determination of the tear constituent was conducted using semi-quantitative estimation of the intensity test lines compared to intensity of a printer picture of color intensities.

Experimental Design:

Subject Population

[000193] Subjects for the study included anyone over the age of 18 years who met the inclusion and exclusion criteria listed in the following tables. The study population included two groups of subjects (Group A, as shown in Table 22, and Group B, shown in Table 23) with approximately equal numbers of each (~ 100 subjects per group):

Table 22: Group A – Healthy Eyes

<i>Healthy Subjects, Inclusion Criteria</i>	
1.	Subject must be 18 years of age and may be of any race and either gender;
2.	The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3.	Subject agrees for samples to be taken from both eyes;
4.	Subject must be willing to follow the study procedures and visit schedule;
5.	Subject must report <2 in all symptoms (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
6.	Subject has at least one of the following in the collection eye(s): <2 in all regions of the cornea (Ora Calibra™ Scale) during visit; - TFBUT > 10 seconds during visit.
<i>Healthy Subjects, Exclusion Criteria</i>	
1.	Subject complained of dry eye or any other acute ocular disease ;
2.	Subject is currently suffering from active inflammation or infection;
3.	Subject used artificial tear drops in the past 2 months;
4.	Subject currently treated medically for a chronic eye syndrome such as glaucoma, allergy or conjunctivitis;
5.	Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or which would present a special risk to the subject;
6.	Subject reports currently being pregnant or nursing;
7.	Use of investigational study drug or study device within 30 days of enrollment.

Table 23: Group B – Suspected Dry Eye

<i>Suspected Dry Eye, Inclusion Criteria</i>
1. Subject must be 18 years of age and may be of any race and either gender;
2. The IRB approved informed consent must be read, signed, and dated by the subject or legally authorized representative. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3. Subject used or had the desire to use artificial tears in the last 30 days;
4. Subject reports ≥ 2 in at least one symptom (Ora Calibra™ Ocular Discomfort & 4- Symptom Questionnaire) during visit;
5. Subject demonstrates both of the following in the collection eye(s): a. ≥ 2 in at least one region (Ora Calibra™ scale) b. TFBUT < 10 seconds during visit;
6. Subject agrees for samples to be taken from both eyes;
7. Subject must be willing to follow the study procedures and visit schedule.
<i>Suspected Dry Eye, Exclusion Criteria</i>
1. Subject is using contact lenses on a regular basis;
2. Subject is currently suffering from active inflammation or infection;
3. Subject has used Restasis® in the last 30 days;
4. Subject used artificial tear drops in the past hour;
5. Subject is being medically treated for glaucoma;
6. Subject has a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or would present a special risk to the subject;
7. Subject reports currently being pregnant or nursing;
8. Subject has participated in any other clinical trial within 30 days of enrollment.

Experimental Design and Methods

[000194] An exemplary embodiment of the method of the present invention was a prospective, single-center, single-visit, parallel-group, data and tear collection study, consisting of approximately 200 subjects. There was one scheduled study visit where subjects were screened; those who met the eligibility criteria were enrolled in the study.

Tear Sample Collection

[000195] The procedure for tear sample collection was as follows:

1. The slit-lamp was set at a low intensity beam.
2. The lower lid of the eye was retracted and a glass capillary tube was placed on the temporal aspect touching the tear surface.
3. The tear surface was contacted and allowed for the collection of between 6-25 microliters of tear solution.
4. Once a sufficient volume (e.g., but not limited to, 6-25 microliters) was collected, the contents of the glass capillary was withdrawn and emptied into a vial. If tear volume was below 6 microliters, a second sample was drawn from the other eye into another clean vial.
5. The vials were marked with a designated subject label provided by the sponsor.
6. The vials were stored at a temperature of 2°C-8° C. Tear samples were transferred to the sponsor laboratories for initial preparation up to 48 hours from collection before further analysis for levels of lysozyme.
7. The tear volume was measured within the 48 hours from sampling using pipette of small volume. Two sample volumes of Phosphate buffer saline (PBS X 1) were added to

collected sample followed by a short vortex (20 Sec.) for mixing. Diluted samples were placed back for storage in a temperature of 2° C -8° C.

Lysozyme Assay

[000196] The assay allows for direct detection of the lysozyme in human tears using specific antibodies that recognize the enzyme. The test strip utilizes semi-quantitative lateral flow immunochromatographic technology. A tear sample is diluted 1:2000 with phosphate saline buffer [i.e., further to the initial 1:3 dilution of the tear] 10 Microliters of sample diluted 1:2000 are placed on the sample pad. Additional 40µL washing solution allows the tear sample to migrate, wetting a conjugate pad. Specific sheep polyclonal antibodies conjugated to gold particles bind the lysozyme. The conjugated antibodies bound to the lysozyme flow through the nitrocellulose membrane. When the gold conjugate/lysozyme complex reaches the test zone, it reacts with a secondary sheep anti-lysozyme antibodies fixated to the membrane surface. A second zone on the nitrocellulose is impregnated (e.g., with goat anti sheep antibodies) and is configured to bind the sheep anti-lysozyme-gold conjugate. A second line forms and is referred to as the Control Line. The control line indicates of test validity. Notably, the two anti-lysozyme antibodies (i.e., a sheep anti-lysozyme or a rabbit anti-lysozyme) can recognize different epitopes on the enzyme.

[000197] In an exemplary embodiment, 1.5mg/ml (0.75-2.5 mg/ml) sheep anti lysozyme was impregnated onto a chromatographic membrane of nitrocellulose with high protein binding capacity (e.g., but not limited to, mdi CNPH-N-5560). Impregnation was visualized by, e.g., but not limited to, the naked eye, as a 1mm wide line. The antibody solution contained the following:

a. Buffer, for example, Phosphate buffer saline at pH 7.4 or Tris, HEPES, Borax or MES buffer

with pH value ranging from 6.5 to 9.0; b. 2% Trehalose (can also be Sucrose), can also range between 1% to 4% sugar; c. 2% ethanol, can also range from 1 to 4%.

[000198] Antibody impregnated nitrocellulose was dried at 50°C for 10 Min to allow the protein fixation to the nitrocellulose. In an embodiment, binding can occur between 60°C and 37°C for 5 to 24 hours, as modulated by temperature (e.g., faster binding at higher temperatures).

[000199] In an exemplary embodiment, sheep anti-lysozyme is conjugated to gold particles (e.g., 20 nm, 40 nm, 60 nm or 100 nm) at a ratio of 4 µg protein per OD1 per ml colloidal gold at 528nm. Conjugation was performed under pH conditions of between pH 7 and pH 9, e.g., pH8.

[000200] An effective concentration of the gold conjugate can range from OD0.5 /ml to OD 2/ml. 30 µg/ml of free sheep anti lysozyme (rabbit anti lysozyme can be used as well) was added to conjugate solution to adjust test sensitivity. Line intensity was estimated (i.e., semi-quantitatively measured) visually as shown in Figure 1. A line intensity of 1 was formed when lysozyme was at a concentration of 25 µg/ml (showing, e.g., equivalence between line intensity and lysozyme concentration). The reaction mix also includes Wash Reagent (WR) that provides chemical surrounding as well as clearing of gold residuals from the nitrocellulose membrane. The WR contains the following: (a) PBS X 1 pH 7.4 (can range from 7 to 9), (b) 1% Bovine Serum Albumin (BSA) can range from 0.5 to 3% and is fatty acid free), (c) between 0.05% and 2% Tween 20, e.g., but not limited to, 0.1% Tween 20, (d) 0.05% N-lauroyl sarcosine and 0.4% PEG to reduce non-specific binding to the nitrocellulose membrane, where the concentration of N-lauroyl sarcosine was from 0.01 to 1%.

Tear Film Break Up Time Test

[000201] The procedure for TFBUT included:

1. A medical professional instilled 5 μ L of 2% preservative-free sodium fluorescein solution into the inferior conjunctival cul-de-sac of each eye. To thoroughly mix the fluorescein with the tear film, the subject was instructed to blink several times. In order to achieve maximum fluorescence, the medical professional waited approximately 30 seconds after instillation before evaluating TFBUT.
2. With the aid of a slit lamp, the medical professional monitored the integrity of the tear film, noting the time it takes to form micelles from the time that the eye was opened. TFBUT was measured in seconds using a stopwatch and a digital image recording system for the right eye followed by the left eye. A Wratten #12 yellow filter was used to enhance the ability to grade TFBUT.
3. For each eye, two measurements were taken and averaged unless the two measurements were greater than 2 seconds apart and were each less than 10 seconds, in which case, a third measurement was taken and the two closest of the three was averaged.

Corneal Fluorescein Staining:

[000202] The procedure for corneal fluorescein included:

1. In order to achieve maximum fluorescence, the medical professional waited approximately 3-5 minutes after instillation before evaluating fluorescein staining. A Wratten #12 yellow filter was used to enhance the ability to grade fluorescein staining.
2. The inter-palpebral was graded and recorded, and the conjunctiva and cornea epithelial were stained by use of a 5 point scale. The upper eyelid was lifted slightly to

grade the whole corneal surface. Regarding conjunctiva, temporal zone grading was performed when the subject looks nasally; grading nasally by looking temporally.

3. The conjunctival and corneal staining was graded and recorded using the Ora Calibra™ Corneal and Conjunctival Staining Scale.

Unanesthetized Schirmer's Test

[000203] The Schirmer Tear Test was performed according to the following procedure:

1. Using a sterile Tear Flo Schirmer's test strip (e.g., obtained from, but not limited to, Rose Enterprises), a bend in the strip was made in line with the notch in the strip.
2. The subject will be instructed to gaze up and in.
3. The Schirmer's test strip was placed in the lower temporal lid margin of each eye such that the strip fits tightly. Subjects were instructed to close their eyes.
4. After 5 minutes have elapsed, the Schirmer's strip was removed. The length of the moistened area was recorded (mm) for each eye.

Ora Calibra™ Ocular Discomfort Scale

[000204] In an exemplary embodiment, ocular discomfort scores were subjectively graded by the subjects according to the following scale, rating each eye separately. The scale used is shown below and ranges from 0-4:

0 = no discomfort

1 = intermittent awareness

2 = constant awareness

3 = intermittent discomfort

4 = constant discomfort

Ora Calibra™ Ocular Discomfort & 4-Symptom Questionnaire

[000205] Subjects rated the severity of each of the following symptoms, with regards to how both their eyes felt, in general – overall ocular discomfort, burning, dryness, grittiness and stinging according to the following 6-point (0 to 5) scale where 0 = none and 5 = worst.

0	1	2	3	4	5
(None)					(Most)

[000206] Standards of professional care to protect the ocular safety of subjects were followed with regard to study regimen adherence. Subjects who met entry criteria provided demographic information, medical and ocular history and artificial tears use if appropriate. Clinical staff confirmed that subjects did not use artificial tears in the hour prior to the study, then guided subjects through the following procedures:

- (1) Subjects completed the OSDI© questionnaire and Ora Calibra™ Ocular Discomfort & 4-symptom Questionnaire.
- (2) Subjects and staff reviewed source documents to confirm that subject met all inclusion/exclusion criteria based on current medications and medical history.
- (3) Clinical staff collected 6-25 microliters of tears using a capillary from the right eye of the subject. Staff labeled the collection vial with the subject screening number and emptied the capillary contents into the vial.
- (4) In the cases where tear volume collected from the right eye was below 6 microliters, a sample was drawn from the left eye and the capillary was emptied into another clean vial marked with same subject screening number.

- (5) Clinical staff performed tear film break up time test on collection eye(s).
- (6) Clinical staff performed Corneal Fluorescein Staining and examined the ocular surface of the collection eye(s).
- (7) If 6 or greater microliters were collected from the right eye, but the subject did not meet Tear Film Break Up Time or Fluorescein Staining inclusion criteria in the right eye, steps 3-6 were repeated in left eye.
- (8) Clinical staff performed un-anesthetized Schirmer's Test on collection eye(s).
- (9) Clinical staff reviewed results to determine if patient met all inclusion/exclusion criteria based on data collected according to items 3-8.
- (10) Patients who met all criteria were assigned a subject study number and categorized on label based on diagnosis of healthy or suspected dry eye patient.
- (11) Adverse events, if applicable, were documented.

[000207] Samples were handled and tested using the following parameters:

- (1) Volume of collected tears was measured using a micropipette. Twice the measured volume was added with Phosphate Buffer Saline (PBS) for a final dilution of 1:3.
- (2) Diluted tears were further diluted serially to the following dilutions: 1:50, 1:100 and 1:200 with PBS.
- (3) Two microliters of diluted sample were mixed with 18 microliters of gold conjugate mix in a microtube. The relevant test strip was dipped in that mix for 4 minutes.
- (4) Additional 25 microliters of wash solution were added to the tube for excess dye clearance from reaction zone.
- (5) After 6 minutes developed test strips were gently blot against tissue paper and scanned with a desk scanner.

(6) Test intensity was quantified according to the intensity scale presented in Figure 1.

Power Analysis:

[000208] Table 24 presents power for selected sample sizes.

Table 24:

Performance Goal	Sample Proportion	N	Power
0.55	0.70	95	0.82
	0.72	75	0.82
	0.75	50	0.84
0.60	0.70	100	0.55
	0.72	100	0.71
	0.75	90	0.84
0.65	0.70	100	0.16
	0.72	100	0.29
	0.75	100	0.55

[000209] The power was estimated using Exact Binomial method, where two co-primary endpoints (sensitivity and specificity) were taken into account, and where “N” represents the number of positive only (or negative only) cases. Thus, the total sample size was doubled.

[000210] Table 25 illustrates a “Precision” parameter, which is defined as a half-length of confidence interval (CI). The CI is an interval estimate of a population parameter. The CI is an observed interval (i.e. it is calculated from the observations), in principle different from sample to sample, that frequently includes the parameter of interest if the experiment is repeated.

Table 25: Confidence Interval Precision

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
80	48	60.0%	48.4%	70.8%	11.2%
	52	65.0%	53.5%	75.4%	11.0%
	56	70.0%	58.7%	79.8%	10.6%
	60	75.0%	64.0%	84.1%	10.1%

Sample Size	Obtained Number of Responders	Obtained Rate	95% Lower CI	95% Upper CI	Precision
	64	80.0%	69.5%	88.2%	9.4%
	68	85.0%	75.2%	92.1%	8.5%
	72	90.0%	81.2%	95.6%	7.2%
90	54	60.0%	49.1%	70.2%	10.6%
	59	65.6%	54.7%	75.3%	10.3%
	63	70.0%	59.4%	79.3%	10.0%
	68	75.6%	65.3%	84.1%	9.4%
	72	80.0%	70.2%	87.7%	8.8%
	77	85.6%	76.5%	92.1%	7.8%
	81	90.0%	81.8%	95.4%	6.8%
100	60	60.0%	49.7%	69.7%	10.0%
	65	65.0%	54.8%	74.3%	9.8%
	70	70.0%	60.0%	78.8%	9.4%
	75	75.0%	65.3%	83.2%	9.0%
	80	80.0%	70.8%	87.4%	8.3%
	85	85.0%	76.4%	91.4%	7.5%
	90	90.0%	82.3%	95.1%	6.4%

Results and Analysis

[000211] The primary outcome of the study was the comparison of benchmark tests for dry eye such as TFBUT, Corneal staining, Schirmer's test, and OSDI questionnaires with results from a test of tear film constituents (e.g., lysozyme).

[000212] All collected samples obtained from patients' eyes which met the entry criteria were included in the analyses. The goal of the study was to develop an assessment tool to compare benchmark tests for dry eye with a kit that tests the tear film compound, lysozyme. Data was distributed from lowest to highest values and compared with other parameters to identify positive and negative correlations. Figure 2 illustrates the correlation of test line intensity with analyte concentration. In some embodiments, a reduced test line intensity correlates with a test for dry eye (e.g., Schirmer's test, corneal staining, OSDI, etc.).

[000213] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by Schirmer's test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by Schirmer's test.

[000214] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by a corneal staining test. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by a corneal staining test.

[000215] In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a lower result as detected by OSDI. In some embodiments, the correlation of the test line indicates that a lower amount of protein (e.g., lysozyme; e.g., but not limited to, 0.1 – 1 µg/mL, 0.1 – 3 µg/mL, 0.1 – 12 µg/mL, 0.1 – 25 µg/mL, etc.) correlates with a higher result as detected by OSDI.

[000216] Sample size in this pilot study (200 total eyes, 100 per group) was not based on any power analysis, but was based on an approximation of the number of eyes sufficient to build a model for a distinguishing between healthy and suspected dry eye tears and evaluation of benchmark standard testing with the different tested parameters.

[000217] Adverse Events (AEs) included any events reported over the course of the tear collection and ocular surface assessment procedures. This clinical study involved TFBUT, corneal staining and the collection of tears for the constituent analysis. During these tests the participant may have felt a foreign body sensation. During the tear collection there may have been cases of direct contact with the eye due to movement, resulting in corneal abrasion, or eye redness. Any such events were noted and graded as follows:

- Mild: Sign or symptom, usually transient, requiring no special treatment, generally not interfering with usual activities.
- Moderate: Sign or symptom, which may be ameliorated by simple therapeutic measures; may interfere with usual activity.
- Severe: Sign or symptom that are intense or debilitating and that interfere with usual activities. Recovery was usually aided by therapeutic measures.

Results

[000218] A total of 198 subjects completed the study, including 126 women and 72 men. The breakdown of subjects according to entry criteria A or B is outlined in Table 26 below. Those who met entry criteria were not matched for age or gender in this study.

Table 26:

	Men	Women	mean age
Group A Healthy	41	59	45.5
Group B Suspected DE	31	67	58.6

Analysis

[000219] Subjects enrolled in each study group met the entry criteria of either healthy or suspected dry eye. The only demographic criteria that showed a significant difference between the two groups was age; preliminary analysis showed no significant difference in any tear metrics between the two groups. In addition, both groups displayed a range of values for the benchmark testing parameters. Based upon this observation, all subjects were pooled into a

single group and analyzed using population quartiles with an assumption that the population sampled represented a continuum of dry eye severity. Using this concept, measurements for each of the benchmark tests were ranked, and mean values for each of 4 quartiles were compared to measures for the tear diagnostics.

Quartile Analysis

[000220] The quartile analysis for TFBUT, inferior staining, and Schirmer's tests are summarized in Table 27. The focus of this approach was on the extremes, quartiles 1 and 4, as these represent those patients with the largest differences for each metric. In all three measures, Q1 was the quartile with values expected for normal patients and Q4 was the quartile with values associated with dry eye disease. For example, those in Q1 have a mean TFBUT of 12.80 seconds and so would be considered normal while those in Q4 have a mean TFBUT of 2.34 seconds, consistent with a diagnosis of moderate dry eye disease. When the mean values for tested parameters in each of the TFBUT-defined quartiles were compared, associations between the break-up time metric and tear constituent dynamics emerged. The decrease in TFBUT between Q1 and Q4 was accompanied by a decrease in lysozyme. Inferior staining increases from Q1 to Q4, and this increase was significantly correlated with an increase in lysozyme. Quartiles defined by Schirmer's scores exhibited significant negative correlations: while the mean Schirmer's score went down from Q1 to Q4, values for lysozyme increases, and showed a significant difference between Q1 and Q4. This negative correlation was due to the nature of the Schirmer's scores, where higher values (Q1) indicated a healthy tear production.

[000221] Table 27 shows quartile analysis for TFBUT, inferior staining and Schirmer's Test. T-test values, where significant (< 0.05), are highlighted **in bold**.

Table 27:

TFBUT				Mean values
	Mean	n	% of eyes	Lysozyme
Q1	12.80	48	24.6%	0.734
Q4	2.34	48	24.6%	0.541
Q4-Q1	-10.46			-0.194
Q1 vs. Q4, t test				<i>0.053</i>
Inferior Staining				
	Mean	n	% of eyes	Lysozyme
Q1	0.42	52	27.5%	0.434
Q4	2.25	59	31.2%	0.757
Q4-Q1	1.83			0.323
Q1 vs. Q4, t test				<i>0.010</i>
Schirmer's Test				
	Mean	n	% of eyes	Lysozyme
Q1	32.44	52	26.40%	0.509
Q4	5.21	52	26.40%	0.797
Q4-Q1	-27.23			0.289
Q1 vs. Q4, t test				<i>0.002</i>

[000222] A second round of quartile analysis used the same approach to determine whether quartiles defined by tear constituent values show similar correlations with other metrics of the signs and symptoms of dry eye disease. These data are shown in Table 28.

Table 28. Quartile analysis for lysozyme. T-test values, where significant (< 0.05), are highlighted in **bold**.

Lysozyme				Mean Values					
	Means	N	% of eyes	TFBUT	OSDI	Ora Calibra Ocular Discomfort	Inferior	Corneal Sum	Schirmer's
Q1	0.00	55	27.78%	5.78	13.66	1.18	1.13	2.48	19.65
Q4	1.07	10	52.53%	6.56	14.77	1.25	1.41	3.18	15.38
Q1-Q4				-0.78	-1.11	-0.07	-0.29	-0.70	4.27
Q1 vs. Q4, t-test				<i>0.307</i>	<i>0.686</i>	<i>0.754</i>	<i>0.025</i>	<i>0.012</i>	<i>0.015</i>

[000223] The lysozyme quartile displayed a significant difference for corneal staining measures, with inferior and total corneal staining showing a positive correlation with increases in protein levels from Q1 to Q4.

Discussion

[000224] The current study illustrated the heterogeneity of the two populations of subjects originally enrolled for analysis. Despite their inclusion based upon differential criteria for symptomology, TFBUT and corneal staining, no significant differences between the two populations were identified in the tear constituent analysis.

[000225] In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis. In some embodiments, the method of the present invention provides for a method of measuring dry eye, including tear constituent analysis, and comparing tear constituent analysis to tests such as, but not limited to, Schirmer's test, TFBUT, etc., so as to obtain information to treat a patient diagnosed with dry eye disease.

[000226] The quartile analyses show the relationships between traditional metrics and the tested parameters which are part of the tear constituents. An exception to this is TFBUT, which shows only modest correlations with any of the measured tear constituents. In contrast, corneal staining measures (such as inferior staining, Table 27) are well-correlated with changes in the tested parameters. This is consistent with a diagnosis of evaporative dry eye, where a reduction in aqueous content of the tears would yield apparent increases in the concentrations of all tear constituents. Alternatively, the increases in tear constituent concentration(s) can result from an inflammatory response to ocular surface distress that initiates a shift in the ratio of serious to mucus lacrimal secretions. Additionally, lysozyme correlates with higher staining and lower Schirmer's scores; however, lysozyme does not show significant correlation with TFBUT.

Example 5: Multi-Assay Analysis of the Tear Film of Healthy and Dry Eye Patients*Tear constituents measured in this study:*

[000227] Lysozyme – Lysozyme is a protein synthesized and secreted by the acini of the lacrimal gland. Published values range from 0.6-2.6 mg/ml in normal tears, where it acts as an antibacterial agent by degrading bacterial cell wall in tear film.

[000228] Lactoferrin – Another protein synthesized by the lacrimal acinar cells, lactoferrin is also present in the concentrations that range from 0.6-3.0 mg/ml. This iron-binding protein inhibits bacterial growth by reducing free iron, and acts as a free radical scavenger.

[000229] Mucin – a glycoprotein synthesized in lacrimal gland, goblet cells and epithelial cells. Multiple forms of mucin are part of the tear film.

[000230] Matrix metalloproteinase 9 (MMP9) – this proteolytic enzyme is synthesized and secreted by inflammatory cells in response to tissue trauma or inflammation.

[000231] Albumin – This serum protein serves as a reporter of increases in capillary and vascular permeability, a common result of inflammation.

Overview

[000232] The objective of this study was to assess the effectiveness of the developed assays in tears of healthy subjects as well as subjects with dry eye, based on the FDA definitions as were used in previous FDA regulatory approval processes for other dry eye syndrome products (Table 29).

[000233] This was a prospective, single center, single visit, parallel group, data and tear collection study. There was one scheduled study visit where subjects were screened and if they met eligibility criteria were enrolled in the study. Source documents served as CRFs for study data collected. There was no test article in this study.

[000234] Written informed consent was obtained from the subject before any procedure specified by this protocol were initiated, including screening procedures. The original signed informed consent forms are maintained with the subject records for all subjects. Standards of professional care to protect the ocular safety of subjects were followed with regard to study regimen adherence.

Selection of Study Population

[000235] The study population was divided into two groups: Group A: subjects with healthy eyes (Control; Approximately 30 subjects); and Group B: subjects with dry eye syndrome (Grades 1-4; Approximately 40 subjects).

[000236] *Inclusion and exclusion criteria:* Inclusion - Subjects must:

1. Be at least 30 years of age and may be of any race and either gender;
2. Be able to read, sign, and date the IRB approved informed consent. Additionally, the informed consent must be signed and dated by the individual consenting the subject;
3. Agree to allow tear samples to be collected from both eyes;
4. Be willing to follow the study procedures and visit schedule;
5. Meet the applicable severity grade criteria of Negative Control, Grade 1, Grade 2, or Grade 3-4;

[000237] Exclusion – Subjects must not:

1. Have an allergy to topical anesthetic or fluorescein dye;
2. Have a history of eye injury, trauma, or ocular surgery within the past 3 months;
3. Have a known blockage of the lacrimal drainage system;

4. Be currently treated medically for a chronic eye syndrome such as glaucoma, allergy or conjunctivitis;
5. Have a condition, which in the opinion of the Principal Investigator, would interfere with optimal participation in the study, or which would present a special risk to the subject;
6. Have worn contact lenses in the last 7 days;
7. Use of investigational study drug or study device within 30 days of enrollment;
8. Have had previous corneal refractive surgery including RK, LASIK, or PRK surgery;
9. Have current active intraocular inflammation or history of intraocular inflammation, e.g. Uveitis.
10. Have used oral doxycycline, corticosteroids, or immunomodulators in the last 30 days;
11. Have received topical ocular corticosteroids, topical ocular nonsteroidal (NSAIDs) therapy, or topical ocular cyclosporine in the last 30 days;
12. Be a female who is currently pregnant or nursing;
13. Have used any topical ophthalmic medications, excluding artificial tears, within 14 days prior to tear collection;
14. Have used any artificial tears within 24 hours of tear collection.

Study Procedures

[000238] *Severity Grading Scheme:* Grading method used to qualify control and dry-eye subjects was based upon the following classification scheme:

Table 29. Dry Eye Grading

Clinical Test	Negative	Mild	Moderate	Moderate/ Severe	Severe
	Control	Grade 1	Grade 2	Grade 3	Grade 4
OSDI score	≤ 13	≥13	≥13	≥13	≥13
TFBUT (sec)	>10	<10	≤ 10	≤ 5	0 ^a
Schimer (mm/5 min)	>10	<10	≤ 10	≤ 5	≤ 2
Staining (0-5 scale)	0	0	1-2	3	≥ 4

^a For Grade 4 TFBUT, 0 = immediate

[000239] *Study Target Enrollment:* The enrollment by subject grades was as follows:

Negative Control: Approximately 30 subjects

Grade 1: Approximately 5 subjects

Grade 2: Approximately 5 subjects

Grade 3-4: Approximately 30 subjects.

Visits and Examinations

[000240] *Visit 1 Procedures:* Baseline and tear collection:

1. Determine whether the subject is willing to participate in the study.
2. Inform subject verbally and in writing (Informed Consent Form) about their participation in the study and provide subject with an appropriate opportunity to ask questions about the study and receive satisfactory answers.
3. Ensure subject reads, signs, and dates the IRB-approved Informed Consent Form prior to any study procedures. In addition, the trained technician should sign and date the informed consent document. The investigator should review the informed consent. The trained technician will then provide the subject with a copy of the signed Informed Consent Form and place the original in the subject folder.

4. Obtain demographic information, medical and ocular history and artificial tears use if appropriate. Make sure subject did not use artificial in the past 24 hours.
5. Instruct the subject to complete the OSDI[®] questionnaire and Ora Calibra[™] Ocular Discomfort & 4-symptom Questionnaire.
6. Perform Visual Acuity.
7. Review source document and confirm the subject meets all inclusion criteria and none of exclusion criteria based on current medications and medical history.
8. Perform Slit Lamp Exam.
9. Perform Meibomian Gland Assessment on both eyes.
10. Collect 6-25 microliters of tears using a capillary from the right eye of the subject.
11. Label vial with the subject screening number; transfer capillary contents to vial.
12. In the case where tear volume collected from the right eye is below 6 microliters, a sample should be drawn from the left eye and the capillary emptied into another clean vial marked with same subject screening number.
13. Perform tear film break up time test on collection eye(s).
14. Perform Corneal Fluorescein Staining and examine the ocular surface on collection eye(s).
15. Perform Unanesthetized Schirmer's Test on collection eye(s).
16. If 6 or greater microliters were collected from the right eye, but the subject does not meet severity grade criteria, repeat steps 10-11, 13-15 in left eye.
17. Review if patient meets all inclusion/exclusion criteria based on data collected.
18. Assign subject study number, record on label based on diagnosis grade.
19. Document any adverse events, if applicable.
20. Fill in Source Document Exit form.
21. Confirm all Source Document visit pages were completed.

[000241] *Visit 2 Procedures:* If a subject's Visit 1 tears cannot be analyzed (Ex. insufficient volume), subjects may be asked to return for a second visit to collect tears. Update medical history/medications/adverse events.

1. Perform Visual Acuity.
2. Perform Slit Lamp Exam.
3. Collect 6-25 microliters of tears using a capillary from the qualified eye(s) of the subject.
4. Fill in Source Document Exit form.
5. Confirm all Source Document visit pages were completed.

Analysis and Safety Variables

[000242] *Tear Measurements:* The sum measures from tears of lysozyme, lactoferrin, matrix metalloproteinase 9, albumin and mucin were analyzed as explanatory variables in a logistic regression to determine association with a grade 1-4 dry eye subjects or healthy subjects.

[000243] Explanatory variables were analyzed in a univariate fashion for association with dry eye. A forward selection procedure was used where after the initial explanatory variable was placed in the model, then additional main effect terms (which were significant within a univariate analysis at a 2-sided alpha = 0.10) would be placed in the model as well as the corresponding two-way interaction terms with the other main effects already in the model, terms were added and kept at a 2-sided alpha = 0.05. If an interaction term met criteria to be added, then the main effect term was also added.

[000244] *Dry Eye Assessments:* Subjects were screened for signs and symptoms of dry eye syndrome as described above.

2. Table 30. Enrolled Subject Demographics

	Healthy Subjects	Dry Eye Subjects			
		Grade 1	Grade 2	Grade 3	Grade 4
<i>Total</i>	30	5	5	33	1
<i>% non-white</i>	10	0	0	0	0
<i>% female</i>	50	100	80	75.6	0
<i>Age, range</i>	31-80	44-63	64-80	39-79	68
<i>Age, mean $\pm$ SD</i>	48.5 $\pm$ 11.4	51.2 $\pm$ 7.6	71.4 $\pm$ 5.9	61.1 $\pm$ 9.3	-

Results

[000245] *Study Subjects:* A total of 74 subjects completed the study, including 5 each classified as Grade 1 or Grade 2 dry eye, 34 subjects with a Grade of 3 or 4, and 30 healthy controls. Demographics are summarized in Table 29. Subjects with dry eye syndrome were more

likely to be female (34/44 for grade 3/4 subjects versus 15/30 for controls) and more likely to be older.

[000246] *Results of Initial Screens:* Results from tear constituent analysis showed that in a Univariate Wald Chi-squared analysis for each, only albumin showed significant ($P < 0.05$) correlation with summated dry eye scores ($P=0.0370$).

[000247] *Tear Analysis Result Modeling:* A total of 74 subjects, 44 with grade 1-4 dry eye and 30 healthy, were included in development of predictive models. As a first step in this process, a predictive algorithm based upon albumin measures was built. The model with albumin alone is:

Table 31. Model 0: Albumin alone

	DF	Standard Estimate	Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	-0.6491	0.5421	1.4338	0.2311
Albumin	1	1.1142	0.5342	4.3506	0.0370

[000248] Using these terms, the probability of being a dry eye (Grade 1-4) subject given tear albumin score is calculated as:

$$\frac{\exp(-0.6491 - 1.1142 * \text{Albumin})}{1 + \exp(-0.6491 - 1.1142 * \text{Albumin})}$$

[000249] After calculating this probability, one then assigns a subject to a group (dry eye or healthy) based on the probability. Using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye $34/44 = 77.4\%$ of time and correctly classifies healthy subjects as healthy $9/30 = 30.0\%$ of the time.

[000250] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $30/44 = 68.2\%$ of time and correctly classifies healthy subjects as healthy $19/30 = 63.3\%$ of the time.

[000251] In a combined model, all variables were entered into the model along with every two-way interaction; a backward selection procedure was implemented to remove terms that were non-significant at a 2-sided alpha = 0.10. If an interaction term met criteria to be added, then the main effect terms were also required. As the number of Hispanic/Latino subjects was small, model fitting was in issue including ethnicity in the model. Therefore, ethnicity and all two-way interactions thereof were removed.

[000252] The resulting model yielded Albumin, Lactoferrin, Age, Gender and Albumin*Lactoferrin as significant explanatory variables and has the following maximum likelihood estimates for the estimating the log odds of the subject being a grade 3/4 dry eye subject:

Table 32 Model 1: Albumin/Lactoferrin + Demographics

	DF	Standard		Wald	
		Estimate	Error	Chi-Square	Pr > ChiSq
Intercept	1	-4.4755	2.2037	4.1247	0.0423
Lactoferrin	1	-10.2477	4.8174	4.5250	0.0334
Albumin	1	-1.9616	1.4646	1.7938	0.1805
Age	1	0.1263	0.0374	11.376	0.0007
gender (F)	1	1.0347	0.3566	8.4180	0.0037
Lactoferrin*Albumin	1	8.7859	4.7024	3.4909	0.0617

[000253] Based upon this model the probability of being a dry eye (G1-4) subject given Albumin, Lactoferrin, Age, and Gender scores is calculated with the expression below:

$$\frac{\exp(-4.4755 - 1.9616 * Albumin - 10.2477 * Lactoferrin + 0.1263 * Age (yrs))}{1 + \exp(-4.4755 - 1.9616 * Albumin - 10.2477 * Lactoferrin + 0.1263 * Age (yrs))}$$

$$\frac{+0.3566 * (-1 \text{ if male}) + 8.7859 * Albumin * Lactoferrin}{+0.3566 * (-1 \text{ if male}) + 8.7859 * Albumin * Lactoferrin)}$$

[000254] After calculating this probability, one then assigns a subject to a group (dry eye or healthy) based on the probability. Using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye $39/44 = 88.6\%$ of time and correctly classifies healthy subjects as healthy $23/30 = 76.7\%$ of the time.

[000255] Increasing the cutoff probability to 55%, the model correctly classifies dry eye subjects as having dry eye $37/44 = 84.1\%$ of time and correctly classifies healthy subjects as healthy $24/30 = 80.0\%$ of the time.

[000256] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $36/44 = 81.8\%$ of time and correctly classifies healthy subjects as healthy $26/30 = 86.7\%$ of the time.

[000257] The results of this model show that choosing either a cutoff probability of 55% or 60% yield sensitivity and specificity $\geq 80\%$.

[000258] Addition of lysozyme alone to Model 1 did not yield any differences in sensitivity or specificity of the model. In contrast, adding interaction terms Lysozyme*Albumin and Lysozyme*Lactoferrin did yield additional predictive power due to the interaction terms, and so a second model was constructed combining all of these terms.

Table 33. Model 2: Albumin/ Lysozyme/Lactoferrin/Demographics

	DF	Standard Estimate	Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	-5.7198	2.9654	3.7204	0.0538
Albumin	1	-3.9059	2.0031	3.8022	0.0512
Lysozyme	1	-0.7375	3.1381	0.0552	0.8142
Lactoferrin	1	-2.7929	5.1812	0.2906	0.5899
Age	1	0.1507	0.0440	11.7043	0.0006
gender (F)	1	1.2206	0.4076	8.9656	0.0028
Albumin*Lactoferrin	1	7.1682	4.4899	2.5488	0.1104
Albumin*Lysozyme	1	4.4090	2.9299	2.2644	0.1324
Lysozyme*Lactoferrin	1	10.7566	7.2803	2.1830	0.1395

[000259] Using a cutoff probability of 50%, the model correctly classifies dry eye subjects as having dry eye $40/44 = 90.9\%$ of time and correctly classifies healthy subjects as healthy $23/30 = 76.7\%$ of the time.

[000260] Increasing the cutoff probability to 55%, the model correctly classifies dry eye subjects as having dry eye $38/44 = 86.4\%$ of time and correctly classifies healthy subjects as healthy $26/30 = 86.7\%$ of the time.

[000261] Further increasing the cutoff probability to 60%, the model correctly classifies dry eye subjects as having dry eye $36/44 = 81.8\%$ of time and correctly classifies healthy subjects as healthy $27/30 = 90.0\%$ of the time.

[000262] The addition of lysozyme and the interactions of lysozyme*albumin and lysozyme*lactoferrin improves the sensitivity and specificity slightly at each cutoff probability.

Safety Results

[000263] There were no reported adverse events or safety concerns in the course of this study.

Discussion and Overall Conclusions

[000264] The purpose of this study was to assess the effectiveness of the developed assays in tears of healthy subjects as well as subjects with dry eye syndrome. First, a standardized grading system was used to define and distinguish populations of healthy subjects from those with different grades of dry eye syndrome. This grading scheme is a composite of four established benchmark tests for assessment of signs and symptoms of dry eye. This definition has been used previously in the U.S. regulatory clinical trial and an FDA approval process of an in-office dry eye screening test called InflammaDry®, a test based upon detection of tear MMP9 levels (Table 29).

[000265] Study subjects graded using the standardized system were also assayed for a panel tear constituents selected based upon their potential to provide an objective measure of dry eye severity. The developed assays results and subjects demographics data were used to build predictive statistical models as a means to judge which developed assays might provide the best diagnostic power.

[000266] Results of the developed assays suggested that albumin was the best assay upon which to base a predictive model, as it showed the highest effectiveness to identify DES subjects. Inclusion of additional assays, however, provides the opportunity for even greater sensitivity and specificity. For this reason, and due to the fact that we know that the DES is a multi-factorial syndrome, we performed all our assays and then combined them in a model to ask the question, given the tear constituent score(s) of each subject, how sensitive and how specific can a combination of these constituents be in terms of their ability to diagnose DES.

[000267] Our test sensitivity represents the number of subjects correctly identified as having DES, while the specificity represents the number of subjects correctly identified as healthy controls. These values can be combined in the positive predictive value (PPV), a measure of what fraction of those subjects identified as DES patients have dry eye. An ideal test would have both a high sensitivity and a high specificity. Table 34 presents a comparison of the sensitivity and specificity of the different models, based upon the results of the different assays.

[000268] As for today there are two main DES diagnostic commercial tests in the market, both related to heterogeneous of the patient population and relaying on a single parameter and trying to diagnose multi-factorial disease - The InflammDry®, a point of use diagnostic that provides a positive or negative assay for the inflammatory marker MMP914 and the TearLab® system which provides a numerical output of tear osmolarity over a range between 302 and 328

mOsm, a range which includes both normal and hyper-osmolar values. The InflammaDry® device and the TearLab® Osmolarity System offer objective diagnostic tests designed for use in the setting of an outpatient office visit; both performed well in sponsored clinical trials.

Table 34. Comparison of Models

MODEL	CUTOFF PROBABILITY	DRY EYE SUBJECTS	HEALTHY SUBJECTS	POSITIVE PREDICTIVE VALUE
		<i>Sensitivity</i>	<i>Specificity</i>	
MODEL 0 albumin	50%	77.4%	30%	72.1%
	60%	68.2%	63.3%	65%
MODEL 1 albumin/lactoferrin + demographics	50%	88.6%	76.7%	79.6%
	55%	84.1%	80%	80.8%
	60%	81.8%	86.7%	86%
MODEL 2 albumin/ lysozyme/lactoferrin/ demographics	50%	90.9%	76.7%	78.8%
	55%	86.4%	86.7%	86.7%
	60%	81.8%	90%	89.1%

[000269] The sensitivity and specificity values from Model 2 (Table 34) are in one line with the commercial diagnostics including the TearLab® Osmolarity system or InflammaDry® (Table 35). This result supports the potential use of Model 2 combined assays as diagnostics for dry eye. Of particular note, the grading scheme for InflammaDry® studies uses the same set of diagnostic criteria for dry eye employed in this study, a major variable in comparisons of different test performance.

[000270] The study results also show that Model 2 is able to diagnose dry eye with sensitivity and specificity superior also to well established existing tests, in particular tests that would normally be conducted in the setting of a clinician's office: Schirmer's Test, TFBUT, symptomatic questionnaires (such as ODSI), or corneal staining.

Table 35. Characteristics of other Dry Eye Tests

TEST (S)	DRY EYE SUBJECTS	HEALTHY SUBJECTS
	<i>Sensitivity</i>	<i>Specificity</i>
SCHIRMER'S TEST	42%	76%
TEAR FILM BREAK-UP TIME	92%	17%
CORNEAL STAINING	63%	89%
QUESTIONNAIRE	89%	72%
INFLAMMADRY [®] (MMP9)	66-97%	97-98%
TEARLAB [®] (OSMOLARITY) ¹⁷⁻¹⁹	64-73%	71-92%

[000271] While both the InflammADry[®] and the TearLab[®] devices have demonstrated good sensitivity and specificity in some trials, in both cases there is debate as to their overall reliability as diagnostics, mainly due to the fact that both tests are related to heterogeneous of the patient population and relaying on a single parameter trying to diagnose multi-factorial disease. For example, several recent studies have concluded that there was no correlation between TearLab[®]-based osmolarity measures and other signs or symptoms of dry eye. Similarly, while the initial assessments of InflammADry[®] rated it with a high sensitivity and specificity, more recent studies found little or no correlation with results from the MMP9 detection device and other dry eye tests. This difference may be attributed to the differences in sample collection methods.

[000272] Both the TearLab[®] system and the current study collect the tear fluid gently from the lateral aspect of the eye. In contrast, InflammADry[®] sampling involves a relatively aggressive rubbing of the lower lid. Direct comparison of MMP9 levels using the two collection methods might be necessary to resolve the basis for the difference in MMP9 findings.

[000273] As a further test of the models derived from this study, a dataset from DiagnosTear first clinical trial, which included suspected healthy and DES patients (recruited according to different inclusion criteria) was tested using Model 2; results are shown in Table 36.

Table 36. Testing Models on First Clinical Trial Results

MODEL	CUTOFF PROBABILITY	DRY EYE SUBJECTS	HEALTHY SUBJECTS
		<i>Sensitivity</i>	<i>Specificity</i>
MODEL 2	50%	73.5%	63%
Results using data from first clinical trial	55%	70.4%	65%
	60%	67.4%	67%

[000274] It may be valuable to test larger populations or other dry eye grading schemes as a test of the models developed in this study. For example, a grading scheme that includes a conjunctival staining component has been used in recent studies of tear protein proteomics.¹⁵ In addition, the sample sized used for the study may introduce a bias due to the age and gender differences in the subject groups, but this is an issue that can be addressed in future tests.

[000275] Inflammation is a known factor in the etiology of dry eye, and tissues exposed to pro-inflammatory signals respond with increases in vascular permeability and exudative fluid loss from the local vasculature. Such exudate can impact the tear film composition with increased electrolyte concentration (i.e., increased osmolarity) and a rise in albumin concentration. Thus, the markers used in this study allow for an integrated measure of several sequela of the dry eye phenotype.

[000276] The use of albumin as a diagnostic has a solid scientific rationale. Albumin diffuses out of dilated conjunctival vessels into the tear film, the concentration of which increases during eye closure and wounding.¹² Tear levels of albumin, therefore, can be considered a marker of ocular surface integrity. In addition, one of the hallmark responses in any inflammatory event is an increase in vascular permeability, and with that increase it is reasonable to expect an increase in the flow of soluble components in circulating plasma (where albumin concentrations range from 3 to 5%) from the vasculature out into the tear film.¹³ The results of

this trial (and other studies) confirm that significant changes in tear film albumin do correlate with dry eye.

[000277] There are no reports to date that demonstrate any clear physiological role for albumin in tears. Despite this, pre-clinical studies of ocular inflammation led Shimura et al (2003) to suggest that albumin in the tear film might represent a compensatory response to reductions in soluble mucins following reduced lacrimation or a loss of goblet cells. The study showed that albumin appears to decrease apoptosis of epithelial cells in rats, suggesting an active role for the serum-derived protein in response to ocular inflammation.¹² They also suggested that tear albumin was a specific marker of ocular surface integrity, a concept that is supported by the findings of DiagnosTear's first clinical study in which a significant positive correlation between albumin and corneal staining was observed.

[000278] The results derived from albumin alone (Model 0) are less robust than those which employ multiple assays, but may benefit from the simplicity of measuring only a single tear component, where the potential for procedural or assays interference issues are minimized. In contrast, it may also be worthwhile to examine the diagnostic power of multi-assays models in subjects with low scores on OSDI surveys who are asymptomatic but meet dry eye criteria based upon staining and other traditional dry eye tests. These subjects are at particular risk for ocular surface damage because of their low levels of discomfort.

[000279] A potential role of lysozyme and lactoferrin in dry eye has been established for some time, as they are known lacrimal gland products and two of the main components of the healthy aqueous phase of the tear film. Levels of these proteins represent a measure of lacrimal gland production and so any alteration in their concentrations in the tear film would imply a

lacrimal gland dysfunction. Other markers in the tears include inflammatory products such as MMP9; such tear markers reflect local, peri-lacrimal infiltration of inflammatory cells.

[000280] To the best of our knowledge, we demonstrate, for the first time, that combination of protein levels originated for a different locations in the eye have a significant ability to diagnose DES. A test that combines changes in one or more of these two tear constituents with albumin will be sampling two distinct physiological responses to ocular surface challenge, and thus may be able to provide a more robust diagnostic output.

[000281] Results from this study confirm that a multi-assay approach is likely to provide the best diagnostic tool for use in the identification and treatment of dry eye syndrome.

[000282] It is appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described hereinabove. Rather the scope of the present invention includes both combinations and sub combinations of various features described hereinabove as well as variations and modifications thereto which would occur to a person of skill in the art upon reading the above description and which are not in the prior art.

[000283] Publications cited throughout this document are hereby incorporated by reference in their entirety. Although the various aspects of the presently disclosed embodiments have been illustrated above by reference to examples and preferred embodiments, it will be appreciated that the scope of the presently disclosed embodiments are defined not by the foregoing description but by the following claims properly construed under principles of patent law.

[000284] In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the presently disclosed embodiments. To the extent that section headings are used, they should not be construed as necessarily limiting.

What is claimed is:

1. A method for diagnosing Dry Eye Syndrome by quantifying an amount of at least two markers in a tear sample collected from a subject, wherein the at least two markers are selected from the group consisting of: Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme, comprising:
 - a. collecting the tear sample containing the amount of the at least one marker from a subject;
 - b. generating a semi-quantitative measurement of the at least one marker by:
 - i. collecting the tear sample containing the amount of the at least one marker from the subject;
 - ii. contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip,
wherein the tear analyzing strip contains an amount of an at least one antibody, or at least one lectin specific for the at least one marker,
wherein the amount of the at least one antibody, or the at least one lectin are configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample;
 - iii. incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker; and
 - iv. utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker,

wherein the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

2. The method of claim 1, further comprising correlating the amount of the at least one marker in a tear sample with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.
3. The method of claim 1, wherein the method quantifies an amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample.
4. The method of claim 3, further comprising correlating the amount of Human Serum Albumin (HSA), mucin, lactoferrin, and lysozyme in a tear sample with a measurement from the group consisting of corneal staining, Schirmer's test, Ocular Surface Disease Index (OSDI) questionnaires, and any combination thereof.
5. The method of claim 1, wherein the tear sample is collected by placing a capillary tube on a temporal aspect of the eye of the subject touching a tear surface.
6. The method of claim 1, wherein the volume of the tear sample is between 0.1 and 25 microliters.
7. The method of claim 1, further comprising calculating the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-0.6491 - 1.1142 * \text{Albumin})}{1 + \exp(-0.6491 - 1.1142 * \text{Albumin})}$$

8. The method of claim 1, further comprising calculating the probability of the subject having dry eye is calculated using the following equation:

$$\frac{\exp(-4.4755 - 1.9616 * \text{Albumin} - 10.2477 * \text{Lactoferrin} + 0.1263 * \text{Age (yrs)})}{1 + \exp(-4.4755 - 1.9616 * \text{Albumin} - 10.2477 * \text{Lactoferrin} + 0.1263 * \text{Age (yrs)})}$$

$$\frac{+0.3566 * (-1 \text{ if male}) + 8.7859 * \text{Albumin} * \text{Lactoferrin}}{+0.3566 * (-1 \text{ if male}) + 8.7859 * \text{Albumin} * \text{Lactoferrin}}$$

9. The method of claim 1, wherein the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.
10. The method of claim 1, wherein the amount of mucin of the tear sample is used to generate a semi-quantitative measurement of mucin by: collecting the tear sample containing the amount of mucin from the subject; contacting the tear sample containing the amount of mucin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of Jacalin bound to biotin and an amount of wheat germ agglutinin (WGA), where the amount of the Jacalin bound to biotin is conjugated to colloidal gold at a ratio of 5 µg/ml Jacalin bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of mucin from the subject on the tear analyzing strip so as to result in a line intensity of mucin; and utilizing the line intensity of mucin to determine the semi-quantitative measurement of mucin; where the semi-quantitative measurement of mucin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

11. The method of claim 1, wherein the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 µg/ml PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.
12. The method of claim 1, wherein the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter

colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

13. A device configured to perform the method of claim 1.

14. A method for calculating the probability of a subject having dry eye, comprising the steps of:

- a. collecting a tear sample containing at least one marker from a subject, wherein the at least one marker is selected from the group consisting of: Human Serum Albumin (HSA), lactoferrin, and lysozyme;
- b. generating a semi-quantitative measurement of the at least one marker by:
 - i. collecting the tear sample containing the amount of the at least one marker from the subject;
 - ii. contacting the tear sample containing the amount of the at least one marker from the subject with a tear analyzing strip,
wherein the tear analyzing strip contains an amount of an at least one antibody or at least one lectin specific for the at least one marker,
wherein the amount of the at least one antibody, or the at least one lectin is configured to generate a line intensity proportional to the amount of the at least one marker present in the tear sample;

- iii. incubating the amount of the at least one marker from the subject on the tear analyzing strip so as to result in a line intensity of the at least one marker;
- iv. utilizing the line intensity of the at least one marker to determine the semi-quantitative measurement of the at least one marker,

wherein the semi-quantitative measurement of the at least one marker is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0; and

- v. calculating the probability of the subject having dry eye is calculated using an equation selected from the group consisting of:

1. $\frac{\exp(-0.6491-1.1142*Albumin)}{1+\exp(-0.6491-1.1142*Albumin)}$; and
2. $\frac{\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))}{1+\exp(-4.4755-1.9616*Albumin-10.2477*Lactoferrin+0.1263*Age(yrs))}$
 $\frac{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}{+0.3566*(-1\text{ if male})+8.7859*Albumin*Lactoferrin}$.

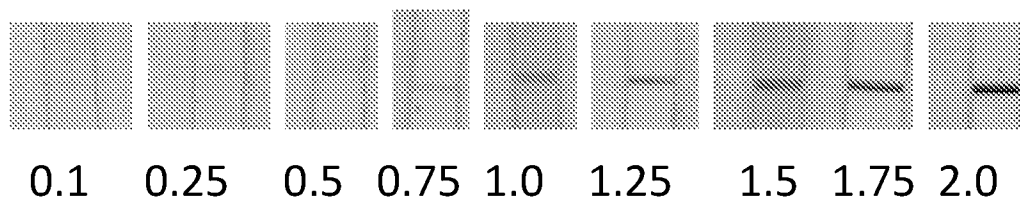
15. The method of claim 14, wherein the amount of HSA of the tear sample is used to generate a semi-quantitative measurement of HSA by: collecting the tear sample containing the amount of HSA from the subject; contacting the tear sample containing the amount of HSA from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of at least one anti-HSA antibody, where the amount of the at least one anti-HSA antibody is conjugated to colloidal gold, incubating the amount of HSA from the subject on the tear analyzing strip so as to result in a line intensity of HSA; and utilizing the line intensity of HSA to determine the semi-quantitative measurement of HSA; where the semi-quantitative measurement of HSA is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

16. The method of claim 14, wherein the amount of lactoferrin of the tear sample is used to generate a semi-quantitative measurement of lactoferrin by: collecting the tear sample containing the amount of lactoferrin from the subject; contacting the tear sample containing the amount of lactoferrin from the subject with a tear analyzing strip, where the tear analyzing strip is bound to an amount of pisum sativum agglutinin (PSA) bound to biotin and an amount of lens culinaris agglutinin (LCA) (where at least the LCA is bound to nitrocellulose of the tear analyzing strip), where the amount of the PSA bound to biotin is conjugated to colloidal gold at a ratio of 5 $\mu\text{g/ml}$ PSA bound to biotin per 1 optical density (OD) per milliliter colloidal gold bound to streptavidin, incubating the amount of lactoferrin from the subject on the tear analyzing strip so as to result in a line intensity of lactoferrin; and utilizing the line intensity of lactoferrin to determine the semi-quantitative measurement of lactoferrin; where the semi-quantitative measurement of lactoferrin is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.
17. The method of claim 14, wherein the amount of lysozyme of the tear sample is used to generate a semi-quantitative measurement of lysozyme by: collecting the tear sample containing the amount of lysozyme from the subject; dilution of the tear sample with a dilution buffer; contacting the diluted tear sample containing the amount of lysozyme from the subject with a tear analyzing strip, where the tear analyzing strip contains an amount of a first antibody (e.g., but not limited to, a sheep or rabbit anti-lysozyme antibody) and an amount of a second antibody (e.g., a rabbit anti-lysozyme antibody), where the amount of the sheep anti-lysozyme antibody is conjugated to colloidal gold at a ratio of 4 micrograms of the sheep anti-lysozyme per 1 optical density (OD) per milliliter

colloidal gold, and the rabbit anti-lysozyme is embedded as capture line on the tear analyzing strip, incubating the amount of lysozyme from the subject on the tear analyzing strip so as to result in a line intensity of lysozyme; and utilizing the line intensity of lysozyme to determine the semi-quantitative measurement of lysozyme; where the semi-quantitative measurement of lysozyme is selected from the group consisting of: 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0.

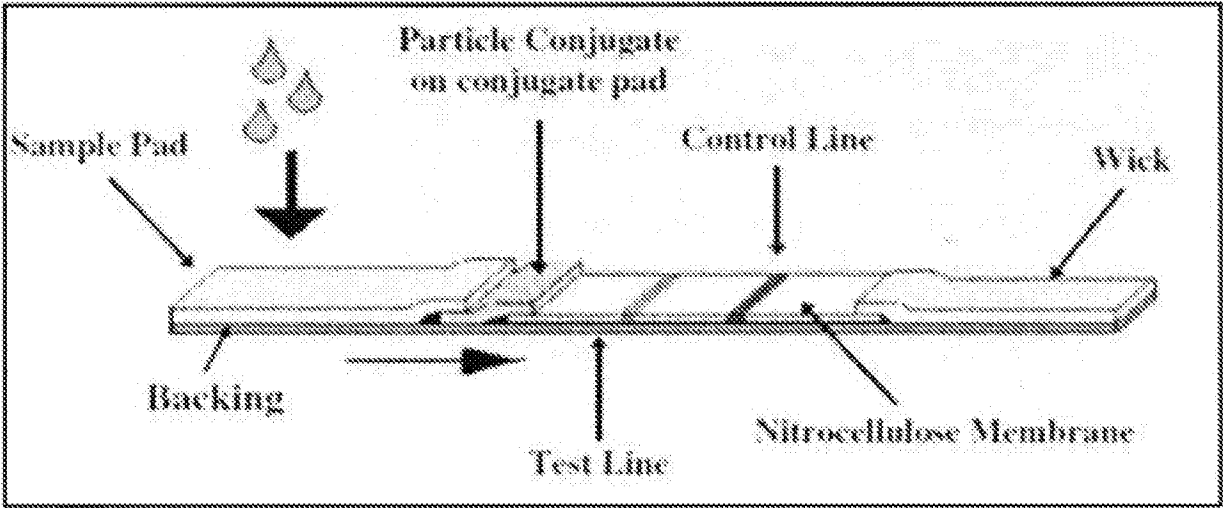
18. A device configured to perform the method of claim 14.

FIGURE 1



Analyte/concentration									
HSAug/ml	0	0.01	0.025	0.05	0.1	0.3	0.5	1.2	4

FIGURE 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB17/00059

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61B 3/02; C12Q 1/06, 1/37; G01N 21/64, 21/77, 33/52, 33/558, 33/577, 33/68 (2017.01)
 CPC - A61B 3/02, 10/0045; C12Q 1/06, 1/37; G01N 21/64, 21/77, 33/52, 33/526, 33/558, 33/577, 33/68, 33/6803

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2012/0171221 A1 (HAMM-ALVAREZ, S et al.) 5 July 2012; figures 1, 7; paragraphs [0044], [0096], [0112], [0121], [0165], [0200]-[0201]; claim 13	1-2, 5-6, 13 — 3-4, 7, 9, 12
Y	US 5,691,207 A (HOLTLUND, J et al.) 25 November 1997; column 11, lines 55-65; column 13, lines 15-40	3-4, 9
Y	(BRON, AJ et al.) Methodologies to diagnose and monitor dry eye disease: Report of the Diagnostic Methodology Subcommittee of the International Dry Eye Work Shop (2007). The Ocular Surface. Vol. 5. No. 2 (2007): 108-152; abstract; table 2; page 114, column 1, paragraphs 1-4	7
Y	(GUERRA, MA et al.) "Quantitative approach for the serodiagnosis of canine Lyme disease by the immunoblot procedure." Journal of clinical microbiology. Vol. 38. No. 7 (2000): 2628-2632; page 2630; column 2, paragraph 2	7
Y	(VIANELLI, A et al.) Bacteriophage T4 self-assembly: localization of gp3 and its role in determining tail length. Journal of bacteriology. Vol. 182. No. 3 (2000): 680-688; page 682, column 1, paragraphs 1-2; page 682, column 2, paragraph 2	12
Y	US 2014/0357971 A1 (DIAGNOSTEAR LTD.) 4 December 2014; abstract; figure 1; paragraphs [0005], [0022], [0031], [0039], [0044]; claim 13	3-4
Y	US 8,323,903 B2 (ARCHER, RA et al.) 4 December 2012; figure 2; column 11, lines 60-67; column 12, lines 1-5; claim 13	12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

21 April 2017 (21.04.2017)

Date of mailing of the international search report

02 JUN 2017

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
 P.O. Box 1450, Alexandria, Virginia 22313-1450
 Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB17/00059

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 8,475,808 B2 (ANDREWS, W et al.) 2 July 2013; abstract; column 10, lines 10-20; column 27, lines 25-40; column 37, lines 5-20; column 38, lines 5-15; claim 26	11
A	US 5,744,096 A (JONES, RM et al.) 28 April 1998; column 9, lines 60-65; column 10, lines 5-20	11
Y	US 5,948,894 A (BERRY, MJ et al.) 7 September 1999; column 12, lines 10-35	12
A	(MARGRAF-SCHONFELD, S et al.) Glycosylation affects ligand binding and function of the activating natural killer cell receptor 2B4 (CD244) protein. Journal of Biological Chemistry. Vol. 286. No. 27 (2011): 24142-24149; figure 6; page 5, column 1, paragraph 3; page 9, paragraph 6	11
A	US 8,377,684 B2 (BAE, BW et al.) 19 February 2013; abstract; column 3, lines 10-25	10
A	US 2010/0068797 A1 (HUBSCHER, TT) 18 March 2010; abstract; paragraphs [0032], [0057]-[0058]. [0092]	10
A	US 5,435,970 A (MAMENTA, EL et al.) 25 July 1995; entire document	1-18
A	WO 2013/162456 A1 (GENERAL ELECTRIC COMPANY) 31 October 2013; entire document	1-18
A	US 2004/0076547 A1 (CARNEY, FP et al.) 22 April 2004; entire document	1-18