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(54) **Title:** LATERAL FLOW ASSAY UTILIZING INFRARED DYE FOR MULTIPLEX DETECTION

(57) **Abstract:** The invention relates to immunoassays for the detection and/or quantification of an analyte in a sample, including a lateral flow assay device for detecting an analyte in a sample comprises a preassembled test strip comprising: (a) a conjugate release pad, (b) a membrane and (c) an absorbent pad, wherein: the absorbent pad, forming a sequential, continuous capillary flow path, the conjugate release pad comprises an absorbed but not immobilized conjugate comprising a first antibody specific for the analyte and conjugated directly to an infrared or near-infrared dye.



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Lateral Flow Assay Utilizing Infrared Dye for Multiplex Detection

Cross-Reference to Related Applications

[0001] This application claims priority to US Ser. No. 61/719,805, filed Oct 29, 2012, the contents of which are incorporated herein by reference.

Background

[0002] The use of point-of-care diagnostics for disease assessment and drug choice is rapidly expanding. Lateral flow assays (LFAs) are popular, point-of-care diagnostics originally developed for in-home measurement of human chorionic gonadotropin for pregnancy determination. LFAs represent a fast, user-friendly diagnostic tool that require little sample pre-treatment, as demonstrated by their use in a variety of tests for drug screening, infection diseases, hormones and cardiac and tumor markers.

[0003] To make the best care decisions, providers in point-of-care settings must be able to rapidly measure multiple analytes in bodily fluids. However, current lateral flow assays used for this purpose are single-plex and read by the eye, thus having limited sensitivity, qualitative results and high subjectivity. To obtain quantitative results, desktop and handheld readers are now being employed and scientists are incorporating a wide range of new detection molecules. While fluorescent nanoparticles, up-converting phosphors, magnetic and electroluminescent-based assays are becoming more popular, they often require complicated antibody-particle conjugation and frequently have issues with consistent particle flow. A simpler solution is to directly attach the antibody to a fluorescent dye, skipping the usage of a particle. However, sensitivity via this method is limited to high ng/mL values due to high-background auto-fluorescence blood proteins and nitrocellulose membrane in the UV and visible spectrums. Furthermore, when simultaneous detection of different analytes present at different concentrations in a fluid sample is very difficult with the current methodologies. Thus, while they are rapid and easy to use, they often lack sensitivity, quantitative output and may be difficult to multiplex.

[0004] LFAs consist of a dried, pre-assembled test strip comprising an immobilized capture and detection reagents (often antibodies) against analytes of interest. Fluid samples activate the test strip as they flow through the material. When capture and detection antibodies recognize conjugate comprising the specific analyte in the fluid sample, a line will form on the membrane of the test strip. The line may be visible to the naked eye as occurs when the detection antibody is bound to colloidal gold, colloidal platinum or dye-filled latex beads. However, these technologies have limited sensitivity, yield qualitative results, and are highly subjective.

[0005] To obtain quantitative results, desktop and handheld readers are now being employed and scientists are incorporating a wide range of new detection technologies. While fluorescent nanoparticles, up-converting phosphors, magnetic and electroluminescent-based assays are becoming more popular, dye-conjugated antibodies (rather than particle-bound antibodies) have seen limited use due to their low signal-to-noise ratio.

[0006] Relevant art: US 5776785 and US 2013/0052669.

Summary

[0007] In an aspect is a lateral flow assay device for detecting an analyte in a sample, the device comprising a preassembled test strip comprising: (a) a conjugate release pad, (b) a membrane and (c) an absorbent pad, wherein: the conjugate release pad overlaps the membrane and the membrane overlaps the absorbent pad, forming a sequential, continuous capillary flow path, the conjugate release pad comprises an absorbed but not immobilized conjugate comprising a first antibody specific for the analyte and conjugated directly to an infrared (IR) or near-infrared (NIR) dye, and the membrane comprises an absorbed, immobilized first stripe of a first capture reagent, and an absorbed, immobilized second stripe of a second capture reagent different from the first capture reagent, wherein the first and second stripes collectively differentiate between analyte-bound and analyte-unbound conjugate.

[0008] In embodiments:

[0009] the device is in a competitive reaction scheme format wherein: the first capture reagent comprises an antigen specific for the first antibody; and the second capture reagent comprises a control antibody specific for the first antibody or the analyte;

[00010] the device is in a direct or double antibody sandwich format, wherein: the first antibody is specific for a first epitope on the analyte; the first capture reagent comprises a capture antibody specific for a second epitope on the analyte different from the first epitope; and the second capture reagent comprises a control antibody specific for the first antibody;

[00011] the sample is blood or blood plasma;

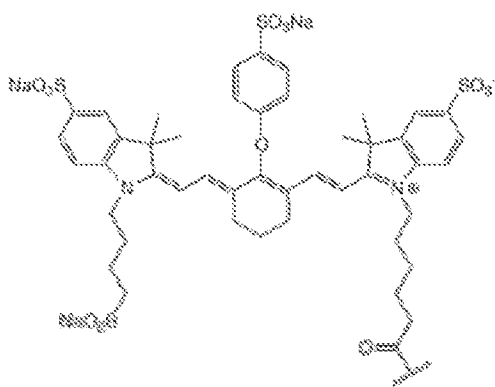
[00012] the dye has maximum excitation and emission peaks in near-infrared, in the range of 680-800 or in the range of 780-800;

[00013] the dye has maximum excitation and emission peaks of about 780 and about 800, respectively;

[00014] the dye is selected from: IRDye® 680, IRDye® 680RD, IRDye® 680LT, IRDye® 750, IRDye® 800CW, IRDye® 700DX, IRDye® 800, IRDye® 800RS, IRDye® 650 Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 (LI-COR®); Cy7 and Cy7.5 (Lumiprobe®); Tracy 645 and Tracy 652 (Sigma-Aldrich®); and Alexa Fluor 647, 660, 680, 700, 750 and 790 dyes (LifeTechnologies®);

[00015] the dye has maximum excitation and emission peaks not substantially diminished in blood or blood plasma;

[00016] the dye is conjugated to the first antibody via an NHS ester reactive group, and wherein the dye has the structure of formula:



I, wherein the wavy line represents the attachment point to the first antibody;

[00017] the analyte is IL-6 or CRP;

[00018] the device further comprises the sample; and/or

[00019] the device further comprises a third stripe of a third capture reagent different from the first and second capture reagents, wherein the third capture reagent is specific for a second analyte of the sample.

[00020] By “and/or” we expressly encompass all combinations of the foregoing particular embodiments as if each combination had been laboriously set forth.

Exemplary such combinations include:

[00021] wherein the sample is blood or blood plasma and the device further comprises the sample;

[00022] wherein the sample is blood or blood plasma, and the dye has maximum excitation and emission peaks not substantially diminished in blood or blood plasma;

[00023] wherein the sample is blood or blood plasma, the dye has maximum excitation and emission peaks not substantially diminished in blood or blood plasma, and the device further comprises the sample;

[00024] wherein the sample is blood or blood plasma; and the dye has maximum excitation and emission peaks in near-infrared, in the range of 680-800 or in the range of 780-800;

[00025] wherein the sample is blood or blood plasma; the dye has maximum excitation and emission peaks in near-infrared, in the range of 680-800 or in the range of 780-800; and the device further comprises the sample;

[00026] wherein the sample is blood or blood plasma; and the dye has maximum excitation and emission peaks of about 780 and about 800, respectively;;

[00027] wherein the sample is blood or blood plasma; the dye has maximum excitation and emission peaks of about 780 and about 800, respectively; and the device further comprises the sample;

[00028] wherein the sample is blood or blood plasma; and the dye is selected from: IRDye® 680, IRDye® 680RD, IRDye® 680LT, IRDye® 750, IRDye® 800CW, IRDye® 700DX, IRDye® 800, IRDye® 800RS, IRDye® 650 Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 (LI-COR®); Cy7 and Cy7.5 (Lumiprobe®); Tracy 645 and Tracy 652 (Sigma-Aldrich®); and Alexa Fluor 647, 660, 680, 700, 750 and 790 dyes (LifeTechnologies®);;

[00029] wherein the sample is blood or blood plasma; the dye is selected from: IRDye® 680, IRDye® 680RD, IRDye® 680LT, IRDye® 750, IRDye® 800CW, IRDye® 700DX, IRDye® 800, IRDye® 800RS, IRDye® 650 Alexa Fluor® 647, Alexa Fluor® 660,

Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 (LI-COR®); Cy7 and Cy7.5 (Lumiprobe®); Tracy 645 and Tracy 652 (Sigma-Aldrich®); and Alexa Fluor 647, 660, 680, 700, 750 and 790 dyes (LifeTechnologies®); and the device further comprises the sample; etc.

[00030] The invention also provides methods for detecting an analyte in a sample using the subject devices, including all embodiments thereof. In embodiments the methods comprise steps: (a) loading the strip with the sample at the conjugate release pad; (b) incubating the device under conditions wherein the analyte of the sample binds the first antibody of the conjugate and the resultant bound analyte migrates by capillary flow through the membrane and is captured at the first or second stripe; (c) irradiating the first and second stripes with IR or NIR light; and (d) detecting a resultant IR or NIR emission at the first or second stripe as an indication of the presence of the analyte-bound conjugate.

[00031] These and other embodiments will be apparent to the skilled artisan based on the disclosure provided herein, including the examples and claims.

Detailed Description of Particular Embodiments

[00032] The technical field of the invention is immunoassays for the detection and/or quantification of analytes.

[00033] The invention employs IR and NIR dyes and IR- and NIR-detection with lateral flow assays (LFAs), including multiplex LFAs. This approach results in a sensitive, flexible assay that can detect an analyte (including detection of different analytes) in test fluids in a fast and reliable manner.

[00034] The LFA devices comprise a conjugate release pad, a component of the device providing a reservoir of the conjugate. In embodiments, the conjugate is applied to the conjugate release pad upon manufacture of the device. For example, the manufacturer applies a solution comprising the conjugate and allows the solution to dry, thereby adsorbing the conjugate onto the conjugate release pad. Such embodiments provide a one-step device by eliminating the need for the user to apply the conjugate to the device. Alternatively, the conjugate material may be applied to the conjugate release pad by the user (e.g., in the form of a solution) immediately before use of the device.

[00035] In embodiments the conjugate release pad is an absorbent material positioned on the backing as described herein. Typically the conjugate release pad is

made of a material that can absorb a large amount of sample, and then release this sample into the membrane at a steady, controlled rate. Such materials are well-known in the LFA art, for example, those provided by WHATMAN®.

[00036] In operation, the user typically applies sample directly to the conjugate release pad. Where the sample comprises the target analyte, the analyte binds to the conjugate, forming an analyte-bound conjugate, on/in the conjugate release pad.

Material flows (e.g., by capillary action) from the conjugate release pad into the membrane. Such material includes the sample fluid, analyte-bound conjugate where present, and analyte-unbound conjugate where present. Analyte-unbound conjugate may be present where there is no analyte in the sample, or where there is an excess of conjugate compared with the amount of analyte in the sample.

[00037] The LFA device comprises a membrane. The membrane is a porous material that provides a path for the flow of material released from the conjugate release pad, and a location for stripes of capture reagents.

[00038] The size of the pores and the porosity of the membrane can vary. Compared with LFAs employing particle-bound dyes and antibodies, the membrane pores of the subject devices are may be smaller. For example, the membrane may contain nominal pore size below 1, 0.5, 0.3, 0.2, or 0.1 μm diameter. In embodiments, the porosity is configured to allow the conjugates to pass/travel through the membrane, but exclude larger microparticles or microspheres.

[00039] Various materials are known and may be used for membranes, such as nitrocellulose, cellulose acetate, glass fibers, and the like.

[00040] The LFA device comprises an absorbent pad. The absorbent pad provides a wicking material to absorb sample after it flows through the membrane (i.e., after passing through any test and control stripes that are present).

[00041] In embodiments the absorbent pad is an absorbent material positioned on the backing as described herein. For example, the absorbent pad is made of a material that can absorb liquid such as the sample at least as rapidly as the sample is released from the conjugate release pad. Such materials are known in the LFA art, for example, those provided by WHATMAN®).

[00042] In an embodiment, the conjugate release pad overlaps the membrane and the membrane overlaps the absorbent pad, forming a sequential, continuous capillary flow path. The overlap is sufficient to ensure contact and continuity between adjacent

components and ensure continuity for the capillary flow path thereby created. For example, the extent of overlap may be 0.5, 1, 2, 3, or 4 mm. In an embodiment, the membrane is a rectangle with a short dimension and a long dimension, and the configuration of the device components is, in order, conjugate release pad, membrane, and absorbent pad arranged linearly along the long dimension of the membrane.

[00043] In embodiments, one or more of the conjugate release pad, membrane, and absorbent pad is/are disposed on a backing, wherein the backing provides additional mechanical strength and stability. For example, the conjugate release pad is disposed on a backing, or the membrane is disposed on a backing, or the absorbent pad is disposed on a backing, or the conjugate release pad and the membrane, or the conjugate release pad and the absorbent pad, or the membrane and the absorbent pad are disposed on a backing, or the conjugate release pad, membrane, and absorbent pad are disposed on a backing. In embodiments, the backing is rigid, such as a glass or ceramic or metal backing. In embodiments, the backing is flexible, such as heavy card-stock or plastic. The backing is optional and may be omitted provided that the device remains mechanically stable (i.e. is not subject to significant damage from normal use and operation) in absence of a backing.

[00044] In embodiments, the backing is provided as a master card, and individual test strips may be cut from the master card. In embodiments, the backing is provided as pre-cut or pre-formed in the appropriate size for an individual test strip. In embodiments, the strips (whether pre-formed or cut from a master) are between 2-20 or 3-10 mm in width, or 3, 4, 5, 6, 7, 8, 9, or 10 mm in width and are between 5-20 or 7-15 cm in length, or 7, 8, 9, 10, 11, or 12 cm in length.

[00045] In embodiments, the backing (when present), conjugate release pad, membrane, and absorbent pad are disposed within a housing. The housing provides further mechanical stability, protects the various components, and improves convenience of the device (e.g., providing hand-holds, increases shelf-life, etc.). The housing may be plastic or any other convenient material. The housing may be shaped similarly to other LFA devices, including having windows for loading sample and reading test results, etc.

[00046] The device is provided in the form of a preassembled test strip. The conjugate release pad, membrane, and absorbent pad are present sequentially (on the

optional backing, when present, and within the housing, when present) to form the continuous capillary flow path.

Analyte and sample

[00047] Analyte detection can be quantitative or semi-quantitative, providing an amount or an amount range or a relative amount of the analyte in the sample.

Alternatively or in addition, detection can be qualitative, providing confirmation of the presence or absence of the analyte in the sample.

[00048] The analyte can be a protein, antigen, virus, biomarker, DNA, RNA, or the like. In embodiments, the analyte has two or more epitopes. In embodiments, the sample comprises two or more analytes of interest, and the LFA device is configured for multiplex detection (i.e., detection of the presence or amount of two or more analytes using a single test device).

[00049] In embodiments, the analyte is a protein, peptide, glycoprotein, interleukin, and the like. Examples included recombinant human IL-6 and recombinant human CRP.

[00050] The sample is a liquid that may be a biological fluid or an aqueous solution, or a combination thereof. For example, the sample may be blood or a blood component such as plasma, or may be blood or a blood component diluted with an aqueous solution (e.g., a buffered solution). In embodiments, the sample is an aqueous solution containing a percentage (1, 2, 3, 4, 5, 10, 15, 20, 25, or more than 25%) of a biological fluid such as blood or a blood component such as plasma. Biological fluids other than blood include urine, saliva, phlegm, amniotic fluid, etc. Non-biological fluids such as lake water and food lots may also provide the sample.

Conjugate

[00051] The conjugate release pad comprises an absorbed but not immobilized conjugate comprising a first antibody specific for the analyte and conjugated directly to an IR or NIR dye. In embodiments, the conjugate is non-particulate (i.e. does not contain a microparticle, nanoparticle, bead, microsphere, nanosphere, or the like).

[00052] The conjugate is delivered to the conjugate release pad via an aqueous detection antibody solution. The detection antibody solution comprises the conjugate and other components provided for solution stability, pH regulation, and the like. Examples of such additional components include buffers, salts, preservatives, etc. Specific examples include BSA, sucrose, trehalose, tween-20, PEG, water (e.g., MiliQ

H₂O), HEPES, Polyvinyl pyrrolidone (PVP), and the like. In an embodiment, the detection antibody solution is applied to the conjugate release pad and allowed to dry for a period of time (e.g., 0.5, 0.7, 1, 1.5 hr) at a specified temperature (e.g., 23, 25, 30, 35, 37, 40 °C). By such application or an equivalent method, the conjugate is absorbed onto the conjugate release pad.

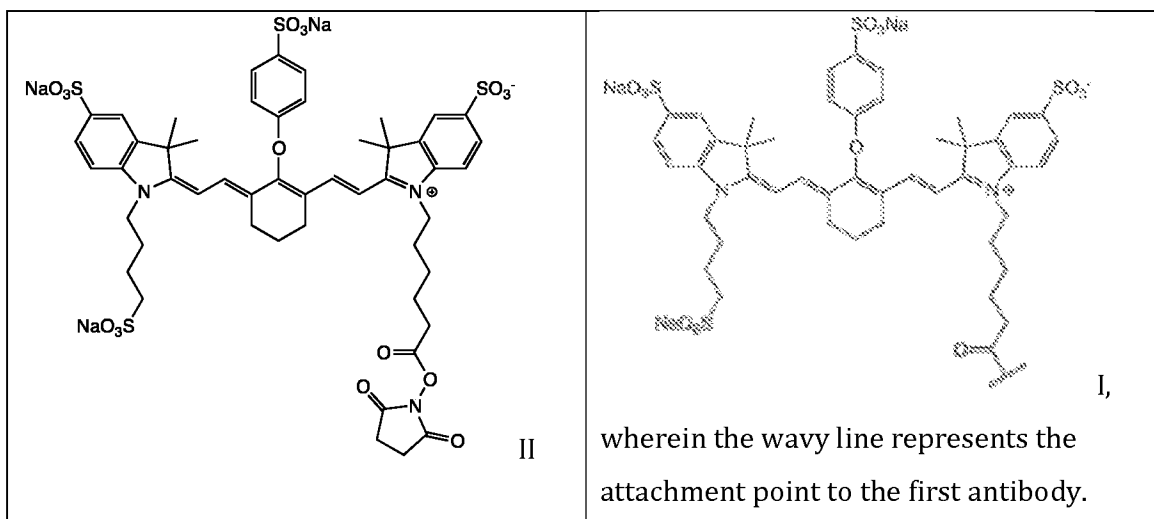
[00053] The conjugate is not immobilized on the conjugate release pad, and can be carried from the conjugate release pad via a flowing solution, such as along a capillary flow path, into the porous membrane.

[00054] The conjugate comprises an IR or NIR dye. These dyes generally have absorption and emission peaks in the 780-2500nm, and 650-800nm ranges, respectively; though the exact peaks are generally dependent on experimental conditions, particularly the solvent. Particularly preferred dyes are NIR dyes with absorption and emission peaks in the range of 680-800, and a wide variety of suitable such Dyes, and particularly NDyes, are well-known, see, e.g. Peng X., Draney D., Near-IR fluorescent dyes for biological applications, LabPlus International, Apr/May 2004, and include: IRDye® 680, IRDye® 680RD, IRDye® 680LT, IRDye® 750, IRDye® 800CW, IRDye® 700DX, IRDye® 800, IRDye® 800RS, IRDye® 650 Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 (LI-COR®); Cy7 and Cy7.5 (Lumiprobe®); Tracy 645 and Tracy 652 (Sigma-Aldrich®); Alexa Fluor 647, 660, 680, 700, 750 and 790 dyes (LifeTechnologies®).

[00055] In particular embodiments the dye is selected so that the dye signal is not substantially diminished (preferably less than 50, 40 or 10%) in blood or blood plasma, and we found absorbance/emission peaks in the 780/800 range particularly suitable for this embodiment, particularly wherein the excitation and emission wavelengths are about 780 nm and 800 nm, respectively.

[00056] In exemplified embodiments the dye is IRDye® 680 (absorption/emission peaks in the ranges 676-680 and 693-700) or IRDye® 800CW (absorbance and emission peaks of 773-778 nm and 789-794 nm, respectively, depending on the solvent, e.g. methanol, water and PBS). 800CW is a conjugated sulfonate salt having (in 1X PBS), and exhibits low background autofluorescence, and low light-scattering. 800CW is obtained as the NHS ester, having the molecular formula C₅₀H₅₄N₃Na₃O₁₇S₄, and having the molecular structure II:

[00057]



[00058] The NHS-group provides a convenient reactive group for attaching the 800CW molecule to an analyte, particularly protein-type analytes. When conjugated to an antibody or other protein having an amine functionality capable of reacting with the NHS-group, the 800CW has the structure of formula (I), supra.

[00059] The conjugate comprises a first antibody specific for the analyte. The identity of the antibody will depend on the specific analyte that is to be the subject of the test. In embodiments, the antibody is a polyclonal antibody such as goat-anti-human IL-6, rat-anti-human IL-6, mouse-anti-human CRP, goat-anti-mouse IgG, and HRP-conjugated Donkey-anti-goat IgG. In embodiments, the first antibody binds to a first epitope on the analyte.

[00060] The dye and the first antibody of the conjugate are conjugated directly to one another. In embodiments, the direct conjugation is via a covalent bond. For example, with an NHS-ester of 800CW, the NHS ester reactive group will label primary and secondary amines, such as lysine residues in proteins (e.g., antibodies). Other methods for conjugating the antibody of the conjugate to the dye will be apparent given the structure of the two components.

First stripe and capture reagent

[00061] The membrane comprises an absorbed, immobilized first stripe of a first capture reagent.

[00062] In embodiments, the device is in a competitive reaction scheme format, and the first capture reagent comprises an antigen specific for the first antibody. In embodiments, the device is in a direct or double antibody sandwich format, in which case the first antibody is specific for a first epitope on the analyte, and the first capture

reagent comprises a capture antibody specific for a second epitope on the analyte different from the first epitope. For example, antibodies and antigens suitable for the first capture reagent include anti-human IL-6, human CRP protein, HRP-donkey-anti-goat IgG, and goat-anti-mouse IgG.

[00063] The first capture reagent is absorbed and immobilized on the membrane. By immobilized (with respect to first, second, and third capture reagents described herein) is meant that the migration of the first capture reagent (e.g., due to capillary flow of fluid such as the sample) from its absorbed location on the membrane is substantially impeded and, in embodiments, completely impeded. Methods for immobilizing the first capture reagent are known in the LFA art.

[00064] The stripe of the first capture reagent is placed on the membrane at a distance from the conjugate release pad. Such distance will depend on the device geometry, and may for example be in the range of 10-50, or 15-35 mm, from the conjugate release pad, or at least 10, 15, 20, or 25 mm from the conjugate release pad. The stripe may be of any suitable width, and determination of such parameter is well known in the art. Example widths include 0.5, 0.8, 1, 1.3, 1.5, or 2 mm.

Second stripe and capture reagent

[00065] The membrane comprises an absorbed, immobilized second stripe of a second capture reagent different from the first capture reagent.

[00066] In embodiments, the device is in a competitive reaction scheme format, and the second capture reagent comprises a control antibody specific for the first antibody or the analyte. In embodiments, the device is in a direct or double antibody sandwich format, in which case the second capture reagent comprises a control antibody specific for the first antibody.

[00067] In embodiments, the second strip functions as a control stripe, and is configured to indicate that the test is properly completed – i.e., that conjugate has reached the second stripe. In such embodiments the first stripe operates as the test stripe to indicate the presence or absence of analyte in the sample. The first stripe is positioned between the conjugate release pad and the second stripe. The amount of space between the first and second stripe may vary according to the device geometry, but will typically be within the range of 5-30 or 10-20 mm, or will be at least 5, 10, or 15 mm.

[00068] The first and second stripes collectively differentiate between analyte-bound and analyte-unbound conjugate.

[00069] In embodiments, the assay consists of a conjugate release pad, membrane, and absorbent pad all attached to a backing. Membranes are striped with a capture reagent at a target line stripe and a control antibody at the control line stripe.

Multiplexing

[00070] In embodiments, the device is configured for multiplex detection of at least two analytes. Configurations for a multiplexed LFA devices are known in the LFA art.

[00071] In embodiments of the multiplexed device, the device comprises a third stripe of a third capture reagent different from the first and second capture reagents, wherein the third capture reagent is specific for a second analyte of the sample. The third stripe may be positioned between the first and second stripes (wherein the first stripe remains closest to the conjugate release pad and the second stripe remains furthest from the conjugate release pad). In such an arrangement, the second stripe can function as a control stripe for both first and third stripes.

[00072] In embodiments, the device may further comprise a fourth stripe of a fourth capture reagent different from the third capture reagent, such as a stripe configured to operate as a control line to indicate that the test is completed and has not malfunctioned. In such embodiments the third and fourth stripes collectively differentiate between analyte-bound and analyte-unbound conjugate. Furthermore, the disclosure herein pertaining to the first and second capture reagents applies equally to the third and fourth capture reagents, respectively.

Format

[00073] The LFA devices herein may be configured for a competitive reaction scheme format, with components described herein. The LFA devices may be configured for a direct or double antibody sandwich format, with components as described herein. In embodiments, the devices are configured with both sandwich and inhibitory assays on the same test strip.

Methods of use

[00074] In embodiments, the devices provide one-step detection of an analyte in a sample, wherein the sample is applied directly to the test, without a pre-mixing step (i.e. a step for combining the conjugate with the sample/analyte) prior to the application. In

other embodiments, it may be desirable to include such a pre-mixing step to improve performance.

[00075] In an aspect is a method for detecting an analyte in a sample using the device as provided herein, the method comprising: loading the strip with the sample at the conjugate release pad; incubating the device under conditions wherein the analyte of the sample binds the first antibody of the conjugate and the resultant bound analyte migrates by capillary flow through the membrane and is captured at the first or second stripe; irradiating the first and second stripes with IR or NIR light; and detecting a resultant emission at the first or second stripe at a IR or NIR wavelength as an indication of the presence of the analyte-bound conjugate.

[00076] In embodiments, the method includes preparation of the sample prior to loading the strip with the sample. Such preparation may include: isolating a fraction from a biological fluid, such as isolating plasma from blood; mixing a biological fluid or fraction thereof with an aqueous solution, such as a buffered and/or stabilized aqueous solution; and concentrating or purifying a biological fluid or fraction thereof. In embodiments, no preparation step is needed, and the sample is used directly without further modification.

[00077] The method involves loading the strip with the sample at the conjugate release pad. The concentration and loading conditions can be easily determined using the disclosure herein and common literature or routine experimentation.

[00078] The method involves incubating the device under conditions wherein the analyte of the sample binds the first antibody of the conjugate and the resultant bound analyte migrates by capillary flow through the membrane and is captured at the first or second stripe. The incubating conditions can be easily determined using the disclosure herein and common literature or routine experimentation.

[00079] The method involves irradiating the first and second stripes with IR or NIR light. The excitation wavelength is determined by the identity of the dye. The irradiation may be carried out with any suitable source such as a laser or the like. The source may be monochromatic or polychromatic provided that the source does not interfere substantially with the detection step.

[00080] The method involves detecting a resultant IR or NIR emission at the first or second stripe as an indication of the presence of the analyte-bound conjugate. The

detecting may be carried out by any suitable detector such as a scanner or camera capable of detecting IR or NIR radiation.

[00081] In embodiments, the excitation and emission wavelengths differ sufficiently such that detection at the emission wavelength is not significantly interfered by operation of the emission source. For example, the excitation and emission wavelengths may differ by 3, 5, 10, 15, 20, 25, 30, 50, 100, or more than 100 nm, wherein such excitation and emission wavelengths are peak wavelengths in the overall emission and excitation spectra of the dye.

Methods of production

[00082] In an aspect is a method for producing the devices herein. The method involves applying the conjugate to the conjugate release pad. The conjugate can be applied in the form of an aqueous solution, in which case the applying can include drying the conjugate release pad under conditions to adsorb but not immobilize the conjugate. The method may further comprise assembling the test strip by overlapping the conjugate release pad, membrane, and absorbent pad as described herein. Such assembly may be carried out on the backing when present. The assembled test strip may be placed within the housing when present. Applying the conjugate to the conjugate release pad can be done after or before assembling the test strip, as appropriate.

[00083] The method of production may further comprise preparing the conjugate by reacting the unconjugated dye with the analyte under conditions suitable for forming a covalent linkage.

Use and Performance

[00084] In embodiments is a one-step multiplex LFA that simultaneously and accurately measures IL-6 and CRP in 10% human plasma on one test strip. The multiplex IR- or NIR-LFA has a detection range of over five orders-of-magnitude and compared favourably to ELISA results.

[00085] In embodiments the device is able to quantitatively “visualize” the concentration of molecules in 10% plasma from concentrations of 10 pg/mL to 1000 ng/mL giving the device a range of over five orders-of-magnitude. Moreover, the operative concentration range of the LFA is “tunable” and can detect molecules present at vastly different (2, 3, 4 or 5 orders of magnitude) concentration in one sample. This feature addresses one critical limiting factor with many assays, therefore allowing

multiplexing for detection of analytes covering a large range of concentrations.

Furthermore, results highly correlate with ELISA results on the same samples and demonstrate intra-assay CVs of less than 5% and inter-assay CVs of less than 10%.

[00086] Therefore, in embodiments, the devices herein provide detection of analytes with lower detection limit compared with conventional devices.

[00087] In embodiments, the devices are useful as a multiplex, quantitative lateral flow assay in a point-of-care setting or in a diagnostic setting. Because the assay format is highly flexible, various analytes can be targeted, including DNA, RNA, lipids, proteins, chemical compound and pathogen concentrations in a variety of samples including blood, plasma, phlegm, saliva, urine, lake water and food lots.

[00088] In one aspect is a dye directly conjugated to antibody as a detection moiety to measure IL-6 and CRP in the LFA format. In an embodiment, an 800CW dye is used for detection in LFAs, providing the benefits of: strong emission under ambient conditions, excitation by cheap and readily available 980 nm lasers, easy conjugation to proteins, and monodisperse avoiding aggregation issues. In an embodiment is a multiplex LFA utilizing both a sandwich and inhibitory assays on the same test strip with a detection range of nearly five-orders-of-magnitude. In embodiments the IR- or NIR-LFA is a one-step test, requires only 7.5 uL of plasma, demonstrates high sensitivity, and correlating with ELISA results. In embodiments is a portable, handheld system with a laser scanner to read the test strips in a point-of-care situation.

[00089] The subject LFAs are superior to fluorescence-based LFAs on several crucial fronts. First, using a longer-wavelength detection dye, it results in reduced background produced by autofluorescent biomolecules and LFA material components. While the fluorescent assays reported have emission/excitation spectrums ranging from UV to 720 nm with most in the 500 to 600 nm range, using a dye in the IR or NIR region helps increase the signal-to-noise ratio in plasma and blood, by reducing the background from autofluorescent proteins and matrix materials, and avoiding possible dampening mediated by hemoglobin absorption.

[00090] Second, while LFAs have classically used particle-based detection methods, the devices herein directly conjugate the dye to the detection antibody. This approach, beside reducing the dye per antibody (as compared to dye-filled latex beads), benefits the assay by providing an easy, fast, and reliable conjugation method that is easily controlled and quantified, therefore making our dyes applicable to a wide range

of assay types using antibodies, proteins, DNA etc. Furthermore, by using dye-conjugated-antibodies rather than particles, particle aggregation on the conjugate release pad and in the membrane itself, is avoided. The subject LFAs show complete release of the antibodies from the conjugate release pad, and strong clearance as the dye-conjugated-antibodies end up mostly on the absorbent pad. Finally, avoiding particles enables us to use a membrane with a smaller pore size, slower flow rate and thus, higher sensitivity.

[00091] Third, the subject LFAs achieve superb sensitivity without requiring pre-mixing steps or washing. Many of the fluorescence-based LFAs reported in the literature pre-mix the detection conjugate with the sample to be analyzed to form complexes before the assay is run. We also avoid the technical issues that arise with drying-down and then rewetting the detection conjugate on the conjugate-release pad. In doing so, it approximates 100% release of the detection conjugate from the release pad. A number of other studies using fluorescence add a wash step following the sample addition to the test strip to facilitate removal of lingering detection conjugates, reducing the test strip background and non-specific binding. Because the assays herein avoid pre-mixing and washing, they are inherently easier to transfer to the real-world marketplace while maintaining the desired sensitivity.

[00092] Fourth, our LFA is easily tuned for different applications. For instance, herein is demonstrated measurement of analyte in 10% human plasma from 10 pg/mL to 2.5 ug/mL – a range of nearly five-orders-of magnitude. This is achieved by employing both a sandwich-based and a competitive-based approach. Additional concentration tuning is easily achieved by other approaches. As an example, the dye-antibody ratio can be easily controlled and optimized with direct dye-to-antibody conjugation. For instance, to escape the hook effect in sandwich-based assays, the addition of fewer to antibodies for detection of large concentrations of analyte allows application of more antibodies to the membrane without affecting the background. Because of the strength of the dye intensity is high, competing, unconjugated detection antibodies may also be added to the release pad to increase the analyte concentration at which a “hook” effect occurs.

[00093] Finally, as a function of the other enhancements, our LFA enables multiplexing of a wide range of analytes at different blood/plasma concentrations. This is exemplified herein by the measurement of IL-6 and CRP on the same test strip, which

exist in the blood at pg/mL and mg/L quantities, respectfully. Previous reports of multiplex detection rely upon conjugate-sample premixing, addition of a wash buffer, and in the case of IL-10/IFN γ , separate mixing and addition of the different detection moieties to the test strip.

[00094] In embodiments, the assay is a duplex IR- and NIR-LFA, comprising additional target and control lines. For example, in embodiments the additional target and control lines are for measurement of CRP. Given the wide range of CRP concentrations in healthy and sick patients, from 0.05 to over 200 mg/L, a competition assay is suitable to avoid complications due to a potential hook effect at high CRP concentrations. Direct or double antibody sandwich formats may be suitable in other circumstances.

[00095] Our LFAs with direct IR-/NIR- detection provide a valuable tool in biomarker evaluation in the point-of-care setting among others. In comparison to LFAs utilizing fluorescent dyes in the ultraviolet and visible spectrum, our LFAs offer superb sensitivity, with detection of proteins in the low pg/mL range. In addition, our LFAs are highly tunable, allowing detection of analytes at concentrations ranging from pg/mL to ug/mL.

[00096] It is to be understood that while the invention has been described in conjunction with examples of specific embodiments thereof, that the foregoing description and the examples that follow are intended to illustrate and not limit the scope of the invention. It will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the invention, and further that other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains. Any combination of the individual embodiments described herein is intended to be part of the invention, as if all such combinations had been laboriously set forth in this disclosure.

Examples: Example 1

[00097] To demonstrate the sensitivity of IR-/NIR-LFAs, a robust LFA was built to measure IL-6 concentrations in 10% plasma. Membranes were striped with rat-anti-IL-6 capture antibody at the target line and mouse-anti-goat capture antibody at the control line. Test strips were cut from a master card to 4 mm width. Goat-anti-IL-6 antibody was conjugated to 800CW dye using an antibody-to-dye ratio of approximately

3. Dye-conjugated antibody was diluted in conjugate-release buffer and 10 uL was applied to the conjugate release pad and dried for 30 minutes at 37 degrees Celsius. Plasma/blood samples were diluted to 10% in running buffer and allowed to test strip up the LFAs. Running time was normally less than five minutes.

[00098] Human plasma samples were spiked with IL-6 from 0 to 500 pg/mL. Samples were diluted to 10% in running buffer and let migrate forward along the membrane. Notably, this was a one-step procedure, as the detection moieties were pre-dried into the conjugate release pad and we did not include a wash step, as this would not be doable in the field. Dried test strips were imaged using the Odyssey Li-cor scanner and images analyzed using Image J. Values were determined as the sum of the gray intensity at the target line divided by the sum of the gray intensity at an area of background directly adjacent to the target line. The LFA for IL-6 was able to readily differentiate values of IL-6 from 10 to 500 pg/mL with signal-to-noise ratios up to 2.0. Moreover, the LFA for IL-6 did not produce a signal when increasing concentrations of mouse IL-6 or human TNF were spiked into the plasma, showing specificity for human IL-6. Overall, the limit-of-detection was 30 pg/mL and the limit-of-quantification was 50 pg/mL for human IL-6.

[00099] The intra- and inter-assay variation were evaluated by mean of spiking plasma with increasing concentrations of IL-6 (0 to 500 pg/mL) on three consecutive days with samples in triplicates. Coefficients of variations (CVs) were then calculated for each concentration of IL-6 (Fig. 3D). CVs over the entire range of IL-6 concentrations remained stable, ranging from 0 to 10%. This is similar to CVs obtained from other IL-6 measurement methods, which normally vary between 0 and 15%.

Example2

[000100] A frequent issue with trying to detect multiple analytes using a single LFA assay is that biomarkers may be present in the test fluid at very different concentrations (from pg/mL to g/mL) and the assays are usually optimized for limited concentration ranges. For example, IL-6 can range between 0.1 and 200 pg/mL while other proteins may be produced at much higher levels.

[000101] CRP is a pentameric blood protein consisting of five, smaller 25,106 Da monomers. CRP is synthesized in the liver in response to inflammation, and is recommended as a biomarker of cardiovascular risk, infection, trauma, tissue necrosis,

autoimmunity and some cancers. Serum from healthy patients normally contains below 10 mg/L. Within this lower range, CRP levels may be divided into quintiles based on the relative risk for future coronary events in men and women. Patients with CRP levels below 0.7 mg/L are in the lowest risk quintile, while patients with levels of CRP between 3.9 and 15 mg/L are in the highest risk quintile. CRP levels above 15 mg/L indicate infection. In fact, during severe bacterial infection CRP levels may increase 50,000-fold up to 200 mg/L.

[000102] Biomarkers are clinically significant molecules that change in relation to disease course. They have numerous uses including aiding diagnosis, predicting future illness, tracking disease flares, predicting responsiveness to pharmaceuticals, and measuring disease response to therapy. IL-6 is a small, 22 kDa cytokine produced by T cells and macrophages in response to infection and after trauma. It mediates fever and the acute phase response, and is required in mice to fight off bacteria. In humans, elevated IL-6 is a biomarker of sepsis, cancer and Alzheimer's disease, among others. While IL-6 is clinically important, it is difficult to measure, as it exists at low concentration in human blood and plasma. IL-6 is normally between 0.1 and 100 pg/mL in human plasma, but during infection can rise to 100 pg/mL. Thus, most LFAs currently available do not have the sensitivity to reliably measure IL-6 concentrations in the blood.

[000103] To evaluate the flexibility of our LFA platform, a multiplex LFA was prepared that can simultaneously measure IL-6 and CRP in human plasma at a 10% dilution. In the development of the duplex assay, the aim was to create an assay that could accurately measure CRP levels in healthy subjects, as well as patients at high risk of cardiovascular events or infection.

[000104] To develop a duplex IR-/NIR-LFA, a target and control line were added for measurement of CRP to the IL-6 LFA described above. Given the wide range of CRP concentrations in healthy and sick patients, from 0.05 to over 200 mg/L, a competition assay format was selected to avoid complications due to a potential hook effect at high CRP concentrations. CRP protein was striped on the membrane 3 mm above the IL-6 capture line and goat-anti-mouse antibody was striped as a control line 3 mm above the IL-6 control line. Using this approach, free CRP present in the sample competes with the striped CRP for binding to the antibody. As the concentration of CRP in the sample increases, the signal directly decreases.

[000105] Developing a one-step, multiplex LFA requires antibody titration and optimization. Concentrations of 800CW-conjugated antibodies against CRP and IL-6 were titrated to achieve a balanced signal intensity. The conjugate release buffer and running buffer were optimized to reduce non-specific-binding, increase signal intensity and remain compatible with future manufacturing goals. Prior blocking of the membrane was found to be unnecessary, as BSA is added to the conjugate release buffer and appears to block non-specific-binding as it flows up the membrane with the sample. Further reduction of NSB wash achieved through alteration of BSA, Tween-20, sucrose, PVA, PEG, etc.

[000106] The multiplex LFA was next tested with diluted plasma samples containing varying concentrations of IL-6 and CRP. IL-6 was titrated from 0 to 80 pg/mL and CRP was titrated from 50 to 2500 ng/mL. These concentrations were chosen to cover the concentration ranges of IL-6 and CRP found in 10% plasma in healthy and sick patients. To determine the degree of interference between IL-6 and CRP, a titration grid was made such that at every concentration of IL-6, there were samples at each CRP concentration. All samples were run in triplicate. The individual concentration curves achieved for IL-6 and CRP while holding the other analyte stable demonstrate that IL-6 and CRP measurements are independent of each other. Further, concentrations curves of IL-6 at all CRP concentrations, and CRP at all IL-6 concentrations have low standard errors. In comparing the IL-6 and CRP curves, IL-6 has more variation across the measurements. However, IL-6 measurements were linear while CRP curves followed power equation.

Example 3

[000107] To enhance the signal-to-noise ratio of fluorescent-dye conjugated antibodies in LFAs, two dyes, 680LT and 800CW, were tested in the near-IR and IR regions with excitation wavelengths above 650 nm. Nitrocellulose membrane were spotted with buffer, dye, plasma and blood at varying concentrations and the auto-fluorescence and signal dampening was measured at excitation/emission peaks of 680/700 and 780/800 nm, respectively. Interestingly, nitrocellulose demonstrated significantly higher auto-fluorescence at 680/700 than at 780/800 nm. Because of this higher background, the ratio of the intensity of the dye spot to the background nitrocellulose, was significantly higher for 800CW. While plasma and blood brightly

auto-fluoresced at 680/700 nm, plasma was nearly invisible at and blood had a dimmer fluorescence at 780/800 nm. When dye was mixed with plasma/blood, both plasma and blood significantly diminished the 680LT signal by nearly 40%, while only reducing the 800CW signal by 5 – 10%. Together, these data indicated that 800CW dye with excitation/emission peaks at 780/800 nm would work best in LFAs with plasma/blood.

Example 4

[000108] Our LFA multiplex assay also performs favorably when compared to the LFA-based tests on the market. With a limit-of-detection of 15 pg/mL for IL-6 in 10% plasma, our LFA shows similar sensitivity as the Milenia® QuickLine IL-6 Rapid Immunochromographic Test which has a limit-of-detection of 50 to 10,000 pg/mL in serum/plasma. However, while the QuickLine IL-6 test requires 100 uL of sample and cannot be used with blood, the LFA is run with only 7.5 uL of plasma/serum and may be utilized with 5% blood without signal dampening. Moreover, as the QuickLine IL-6 test cannot measure analyte over 0.5 mg/L due to a hook effect, it may not be applicable for multiplexing with CRP. The LFA utilizes an inhibitory-style test to measure CRP (in 10% plasma) from 0.050 to 2.5 ug/mL which represents 0.5 to 25 mg/L in whole blood. This allows for measurement of CRP in healthy people and differentiation of people that are at higher risk to develop cardiac risk. Further, because of the competitive ELISA principle, samples with CRP levels above 25 mg/L (patients with severe infections) will be readily determined as the test will show no signal. Thus, our IR-/NIR-LFA compares favorably to the NycoCard CRP test (Axis-Shield), which has a range of 5 to 120 mg/L for serum/plasma samples and involves multiple steps.

Example 5

[000109] **Reagents and apparatus.** Antibodies purchased included polyclonal goat-anti-human IL-6 (R&D Systems), rat-anti-human IL-6 (BD Biosciences), mouse-anti-human CRP, goat-anti-mouse IgG, and HRP-conjugated Donkey-anti-goat IgG. Proteins purchased included recombinant human IL-6 (R&D Systems) and recombinant human CRP. Buffer reagents including bovine serum albumin (BSA), sucrose, trehalose, sodium azide, tween-20, sodium chloride (NaCl) and Polyvinyl Pyrrolidone were purchased from Sigma. HEPES was purchased from Gibco and phosphate buffer was attained from Calbiochem.

[000110] **Antibody dye conjugation.** Antibodies were conjugated to 800CW dye via an NHS ester reactive group following standard protocols indicated in the IRDye® 800CW Microscale Protein Labeling Kit (Li-Cor Biosciences). Briefly, antibodies were diluted to 1 mg/mL in 50 mM phosphate buffer, pH 8.5. Potassium phosphate buffer was added at a ratio of 1:10 to increase the pH, dye was added at a dye to protein molar ratio of 3:1, and contents were reacted for 2 hours at room temperature. To purify antibodies conjugated 800CW dye from free 800CW dye, low molecular weight molecules were removed via a 40 kDa desalting spin column (Pierce Zeba). Final antibody concentration was evaluated via a BCA protein assay kit (Pierce), and the number of dye to antibody ratio was determined by measurement of absorption at 780 nm as compared to a standard curve.

[000111] **LFA preparation.** Capture reagents were diluted in buffer containing 10 mM HEPES, 135 mM NaCl, 2% BSA and 0.02% sodium azide. Antibodies and protein were striped on a HF240 membrane (Millipore) at 1 uL/cm using an Ivek MicroStriper II. Capture reagents were striped at the following specified distances past the conjugate release pad: 500 ug/mL of anti-human IL-6 at 15 mm, 1 mg/mL of human CRP protein at 18 mm, 10 ug/mL of HRP-donkey-anti-goat IgG at 30 mm, and 10 ug/mL of goat-anti-mouse IgG at 33 mm. Striped membranes were dried at 37 °C for 30 minutes and stored in a desiccator at 4 °C until used.

[000112] The striped membrane, conjugate release pad and absorbent pad were assembled together on the mast card with 2 mm overlapping between each component using a Kinematic Automation Matrix 2210 Universal Laminator Module. The assembled card was cut into 4 mm wide strips with a Kinematic Automation Matrix 2360 Programmable Shear. 800CW-conjugated detection antibody was diluted to specified concentrations in dilution buffer consisting of 10 mM HEPES, 5% BSA, 5% Trehalose, 1% Polyvinyl Pyrolidone (PVP) and MiliQ H2O. The buffer had previously been optimized by altering the concentrations of salt, BSA, sucrose, tween-20 and PEG. 10 uL of the detection antibody solution was applied to the conjugate release pad of each test strip and dried for 1 hr at 37 °C.

[000113] **LFA assay protocol.** Human plasma collected in EDTA tubes (Stanford Blood Bank) was diluted to 10% final concentration in a running buffer consisting of 10 mM HEPES, 135 mM NaCl, 0.5% Tween-20 and 0.02% sodium azide. 75 uL of the plasma buffer mixture was applied to the release pad of each test strip and allowed to

run until completion. Test strips were either read wet after 10 minutes running or allowed to dry to completely and read. All samples were run in triplicate.

[000114] **Image acquisition and data analysis.** Test strips were visualized by placing them face-down on an Odyssey Li-cor Scanner. Test strips were scanned only for emission at 800 nm with an intensity level of 5, a 3 mm focus offset and 169 um resolution. Collected images were exported as tiff files and analyzed using Image J software (NIH). Briefly, the raw integrated density inside rectangular areas was determined at the lines of interest and 1 mm in front of the lines of interest to determine background intensity. All data was then imported into excel for analysis.

[000115] For each analyte on each test strip, the ratio of the intensity of the capture line to the intensity of the adjacent background was determined. For each analyte, a standard curve using known concentrations of analyte was pre-determined. Intensity ratios for unknown samples were then compared to the standard curve to determine analyte concentrations.

[000116] **ELISAs.** Plasma from twenty-five human subjects was analyzed by ELISA for human IL-6 (R&D Systems) and human CRP. All samples were run in triplicate. For the ELISA, sample intensities were compared directly to a standard curve accompanying the kit.

[000117] Individual ELISAs are often used to measure concentrations of IL-6 and CRP in human plasma. To compare results obtained from our LFAs to ELISAs, the amount of IL-6 and CRP was measured via ELISA in the same samples that were applied to the LFAs above. Undiluted samples were added to IL-6 ELISA wells, while samples were diluted 500 to 3000 fold in assay diluent to measure CRP concentrations via ELISA. To compare results, the IL-6 and CRP values were calculated in pg/mL and ng/mL, respectively, from the intensity ratio for every sample using standard curves. The mean value of triplicates were graphed for every sample as measured by ELISA or LFA. Impressively, LFA and ELISA results were strongly correlated, showing R^2 values of 0.92 and 0.95 for IL-6 and CRP, respectively. The equations of correlation were $y=1.16939*x-4.1834$ and $y=1.0602*x-47.035$ for IL-6 and CRP, respectfully.

[000118] To determine the agreement between our LFAs and ELISAs, Bland-Altman residual plots were constructed. The limits of agreement (bias +/- 1.96SD) were calculated as 18.9 and -17.4 pg/mL, and 342 and -348 ng/mL for IL-6 and CRP, respectively. From these plots, NIR-LFAs and ELISAs show strong agreement with few

samples outside the limits of agreement. Further, strong biases away from the mean are not seen for our IR-/NIR-LFAs.

[000119] The CVs obtained were compared via LFAs and ELISAs. CV values were similar for our IR-/NIR-LFA and ELISA results. For both methods, CVs for IL-6 measurements were below 10%. For CRP, CVs were higher with both methods, rising to 17.6% as seen with the ELISA.

[000120] **Statistics.** Coefficients of variation were calculated as the standard deviation divided by the mean. For differentiation of samples, two-tailed student's t-tests were performed.

[000121] In summary, an LFA system was developed that takes advantage of the beneficial properties of IR and NIR dyes. Our LFA employs detection antibodies directly conjugated to dye, rather than to particles. This allowed the use of a membrane with a smaller pore size, which ran slower and thus, had a higher sensitivity. As compared to other detection methods, our LFA demonstrates comparable sensitivity to classical IL-6 ELISAs, and better sensitivity than LFAs reported in the literature and commercial LFAs. Moreover, although the IR-/NIR-LFA has low picomolar sensitivity, its analyte detection range can easily be tuned to fit the needs of the assay. Efficacy was demonstrated by analyzing two proteins at extremely different concentrations in the blood – IL-6 and CRP – on the same test strip. It was found that IL-6 and CRP can be measured on the same test strip, with no interference between the 2 analytes,. With such duplex LFA there was measured the IL-6 and CRP concentrations human plasma with 42 different mixtures of IL-6 and CRP added. The duplex LFA produced results similar to the ELISA results, having R-squared values of 0.92 and 0.95 for IL-6 and CRP, respectfully. When experimental variation was examined, it was found that the duplex LFA had CVs comparable to ELISA, with less than 10% variation for IL-6 measurements and less than 20% variation for CRP measurements.

[000122] The invention encompasses all combinations of recited particular and preferred embodiments. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein, including citations therein, are hereby incorporated by reference in their entirety for all purposes.

What is claimed is:

1. A lateral flow assay device for detecting an analyte in a sample, the device comprising a preassembled test strip comprising: (a) a conjugate release pad, (b) a membrane and (c) an absorbent pad, wherein:

the conjugate release pad overlaps the membrane and the membrane overlaps the absorbent pad, forming a sequential, continuous capillary flow path,

the conjugate release pad comprises an absorbed but not immobilized conjugate comprising a first antibody specific for the analyte and conjugated directly to an infrared or near-infrared dye; and

the membrane comprises an absorbed, immobilized first stripe of a first capture reagent, and an absorbed, immobilized second stripe of a second capture reagent different from the first capture reagent, wherein the first and second stripes collectively differentiate between analyte-bound and analyte-unbound conjugate.

2. The device of claim 1 in a competitive reaction scheme format, wherein:

the first capture reagent comprises an antigen specific for the first antibody; and

the second capture reagent comprises a control antibody specific for the first antibody or the analyte.

3. The device of claim 1 in a direct or double antibody sandwich format, wherein:

the first antibody is specific for a first epitope on the analyte;

the first capture reagent comprises a capture antibody specific for a second epitope on the analyte different from the first epitope; and

the second capture reagent comprises a control antibody specific for the first antibody.

4. The device of claim 1 wherein the sample is blood or blood plasma.

5. The device of claim 1 wherein the dye has maximum excitation and emission peaks in near-infrared.

6. The device of claim 1 wherein the dye has maximum excitation and emission peaks in the range of 680-800.

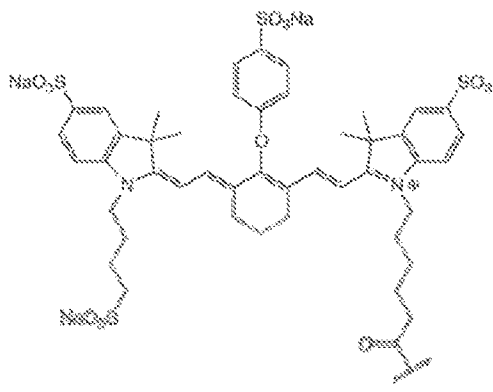
7. The device of claim 1 wherein the dye has maximum excitation and emission peaks in the range of 780-800.

8. The device of claim 1 wherein the dye has maximum excitation and emission peaks of about 780 and about 800, respectively.

9. The device of claim 1 wherein the dye is selected from: IRDye® 680, IRDye® 680RD, IRDye® 680LT, IRDye® 750, IRDye® 800CW, IRDye® 700DX, IRDye® 800, IRDye® 800RS, IRDye® 650 Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 (LI-COR®); Cy7 and Cy7.5 (Lumiprobe®); Tracy 645 and Tracy 652 (Sigma-Aldrich®); and Alexa Fluor 647, 660, 680, 700, 750 and 790 dyes (LifeTechnologies®).

10. The device of claim 1 wherein the dye has maximum excitation and emission peaks not substantially diminished in blood or blood plasma.

11. The device of claim 1 wherein the dye is conjugated to the first antibody via an NHS ester reactive group, and wherein the dye has the structure of formula:



wherein the wavy line represents the attachment point to the first antibody.

12. The device of claim 1 wherein the analyte is IL-6 or CRP.

13. The device of claim 1 further comprising the sample.
14. The device of claim 1, further comprising a third stripe of a third capture reagent different from the first and second capture reagents, wherein the third capture reagent is specific for a second analyte of the sample.
15. A method for detecting an analyte in a sample using the device of claim 1, the method comprising steps:
- (a) loading the strip with the sample at the conjugate release pad;
 - (b) incubating the device under conditions wherein the analyte of the sample binds the first antibody of the conjugate and the resultant bound analyte migrates by capillary flow through the membrane and is captured at the first or second stripe;
 - (c) irradiating the first and second stripes with IR or NIR light; and
 - (d) detecting a resultant IR or NIR emission at the first or second stripe as an indication of the presence of the analyte-bound conjugate.

A. CLASSIFICATION OF SUBJECT MATTER**G01N 33/53(2006.01)i, G01N 33/558(2006.01)i**

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

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
G01N 33/53; G01N 33/52; G01N 33/58; G01N 33/72; G01J 1/58; G01N 33/68; G01N 33/558Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility modelsElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: lateral flow assay device, infrared, near-infrared, antibody, dye**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011-137388 A2 (FRED HUTCHINSON CANCER RESEARCH CENTER) 3 November 2011 See abstract and claims 1, 2, 3, 9, 15, 16.	1-15
A	MARSHALL et al. 'Single-dose intravenous toxicity study of IRDye 800CW in Sprague-Dawley rats' Molecular Imaging and Biology, Vol.12, No.6, pp.583-594 (8 April 2010) See abstract, page 584 and figure 1.	1-15
A	KR 10-0963563 B1 (BORE DA BIOTECH CO., LTD.) 15 June 2010 See abstract, claim 1, page 5 and figure 1.	1-15
A	KR 10-0704007 B1 (KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY) 4 April 2007 See abstract, claims 1, 3, 4 and figure 1.	1-15
A	US 2009-0289201 A1 (BABU et al.) 26 November 2009 See abstract, claims 1, 2, 9 and paragraphs [0029], [0030].	1-15
PX	SWANSON et al., 'Lateral flow assay with near-infrared dye for multiplex detection' Clinical Chemistry, Vol.59, No.4, pp.641-648 (30 January 2013) See abstract.	1-15



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

26 February 2014 (26.02.2014)

Date of mailing of the international search report

03 March 2014 (03.03.2014)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/067156

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011-137388 A2	03/11/2011	WO 2011-137388 A3	08/03/2012
KR 10-0963563 B1	15/06/2010	None	
KR 10-0704007 B1	04/04/2007	None	
US 2009-0289201 A1	26/11/2009	US 8470608 B2	25/06/2013