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(54) Title: METHODS FOR DETECTING A BIOLOGICAL MOLECULE

(57) Abstract: Provided herein are methods relating to the detection of an analyte in a sample. Methods of detecting and/or quantifying insulin in a sample, including a biological sample are described. Kits and devices related to detecting insulin are described.



## Methods for Detecting A Biological Molecule

### Technical Field

[0001] Methods relating to the detection of an analyte in a sample. In particular, disclosed are methods of detecting and/or quantifying insulin in a sample, including a biological sample. Methods, kits, and devices related to detecting insulin are described.

### Background

[0002] Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

[0003] Insulin is a major hormone produced in the pancreas which regulates glucose metabolism in the body. It is essential for facilitating the uptake of glucose from the food into the cells, where glucose is broken down to produce the energy required for cells to work properly.

[0004] High insulin levels are a major cause of obesity, diabetes, cardiovascular disease and may also increase the risk of breast cancer and infertility. For example, diabetes is a progressive chronic disease where the pancreas does not produce enough insulin, or the body becomes resistant to the normal effects of insulin. To compensate for this, the insulin-resistant individual's pancreas releases large amounts of insulin so that enough cells are stimulated to absorb glucose. This usually leads to a sharp drop in blood glucose levels and a hypoglycemic response several hours after a meal. Therefore, the rapid and accurate monitoring of insulin levels in individuals will enable them to verify if their diets and lifestyles are healthy, providing immediate positive reinforcements, and is also crucial for early detection of various chronic diseases and management of personal health conditions (D. Melloul, S. Marshak and E. Cerasi, *Diabetologia*, 2002, 45, 309-326).

[0005] The common clinical methods for insulin detection include enzyme-linked immunosorbent assays (ELISA) (Y. Kumada et al., *Journal of Biotechnology*, 2007, 127, 288-299), and immunoradiometric assays (M. Deberg et al., *Clinical Chemistry*, 1998, 44, 1504-1513). Although the results from these detection methods are reliable, the detection process is complicated, time-consuming and expensive. These methods require a blood draw and must be carried out in a pathology laboratory by a qualified person. Further, immunoradiometric assays require radioactive elements which are harmful to the human body.

[0006] For basic research purposes, insulin detection has been performed using various methods including flow injection analysis, high performance liquid chromatography, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, fluorescence,

electrochemiluminescence and electrochemistry. Although these methods provide sensitive detection of insulin, they suffer from several disadvantages such as time-consuming steps and the requirement of high volume of reagents and biological samples, sophisticated instruments and technical skills. Accordingly, the detection strategies outlined above are not suitable for point-of-care diagnostics.

[0007] There is a long-standing problem in the field of diagnostics that biological molecules such as insulin in samples, cannot be detected with sensitivity and specificity without expensive and time-consuming methods requiring highly trained personnel.

[0008] It is an object of the present disclosure to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

## Summary

[0009] Methods of detecting insulin in a sample comprising an aptamer specific for insulin and a lateral flow assay device (LFD) are described. In certain embodiments, the methods comprise qualitatively detecting insulin in a biological sample. In certain embodiments, the methods comprise quantitatively detecting insulin in a biological sample.

[0010] Methods described herein relate to detecting insulin comprising applying a sample to a lateral flow assay device. As a "lateral flow" assay device, the device is configured to receive a sample at a sample region and to provide for the sample to move laterally, via, e.g. wicking, by capillary action from the sample region to a detection region.

[0011] Provided are methods, kits, and devices for the detection of insulin in a sample. The methods and kits relate to a lateral flow assay device (LFD) for the detection of insulin in a sample, including a biological sample. Biological samples from which insulin may be detected by application of the methods, kits, and devices herein described include, but are not limited to, blood, serum, saliva, urine, or exhaled breath condensate. Biological sample may be applied directly to a sample region of a LFD herein described without any preparation. In embodiments herein described, blood directly from, for example, a pin pricked finger, may be applied directly to a sample region of a LFD herein described. In embodiments herein described, saliva collected from a subject may be applied directly to a sample region of a LFD herein described. In embodiments herein described, exhaled breath condensate collected from a subject may be applied directly to a sample region of a LFD herein described.

[0012] Embodiments of a lateral flow assay device (LFD) describe here include a competitive assay-based LFD (LFD related to the first aspect), a Rolling Circle Amplification (RCA)-based LFD (LFD related to the second aspect), and a liposome signal amplification-based LFD (LFD related to the third aspect) paired with a glucose meter for signal readout.

[0013] The signal readout on an LFD herein described may be monitored by personal equipment, including but not limited to a smartphone or a glucose meter. The methods, kits and devices provided herein offer detection and point-of-care monitoring of insulin. Provided are portable lateral flow assay devices as described herein for the detection of insulin in a sample. The methods, kits and devices provided herein allow patients to monitor their health conditions at home and share these data with their doctors.

[0014] The methods, kits and devices provided herein are particularly advantageous in the early diagnosis and management of chronic diseases such as diabetes.

[0015] Detection of insulin in a sample using a lateral flow assay device described herein may comprise assessing a test line to quantify a concentration of insulin in the sample. In certain embodiments, the test line comprises a component that decreases in concentration as insulin concentration increases in a sample.

[0016] In a first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component;
- (ii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe;

and;

- (iii) an absorbent pad.

[0017] In one embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component;
- (iii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe;

and;

- (iv) an absorbent pad.

[0018] In one embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TT-3'; and wherein the detection component comprise gold nanoparticles (AuNPs);
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe comprising a sequence of 5'-AAA AAA AAA AAA AAA AA-3'; and
    - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';
- and
- (iv) an absorbent pad.

[0019] In one embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise the fluorescence dye Texas red;
  - (iii) a detection region comprising:
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe comprising a sequence of 5'-AAA AAA AAA AAA AAA AA-3'; and
    - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';
- and
- (iv) an absorbent pad.

[0020] Detection of insulin in a sample using a lateral flow assay device described herein may comprise assessing a test line to quantify a concentration of insulin in the sample. In certain embodiments, the test line captures a component that increases in concentration as the insulin concentration increases in a sample.

[0021] In a second aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising:
    - (a) an insulin probe comprising an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); and
    - (b) a control component;
  - (ii) a detection region comprising:
    - (a) a control line comprising a control component binding molecule; and
    - (b) a test line comprising a target component binding molecule;
- and;
- (iii) an absorbent pad.

[0022] In one embodiment related to the second aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO);
  - (ii) a conjugation pad comprising a control component;
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule; and
    - (b) a test line comprising a target component binding molecule;
- and;
- (iv) an absorbent pad.

[0023] In one embodiment related to the second aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3'; and wherein the target component is fluorescein (FAM);

- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad.

[0024] In embodiments related to the lateral flow assay device of the second aspect, provided are methods of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a sample comprising

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3'; and wherein the target component is fluorescein (FAM);
- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad;

wherein the method comprises the steps of

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) applying a RCA reaction mixture to the detection region of the LFD, wherein the RCA reaction mixture comprises
  - (1) a circular RCA template comprising the sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3';
  - (2) a mixture of dNTPs;
  - (3) a DNA polymerase, wherein the DNA polymerase is phi 29 DNA polymerase;

- (4) a detection component, wherein the detection component is 3,3',5,5'-tetramethylbenzidine (TMB); and
- (5) hemin;
- (d) incubating the LFD;
- and
- (e) assessing the intensity on the test line.

[0025] In embodiments related to the lateral flow assay device of the second aspect, provided is a method of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a sample comprising

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3'; and wherein the target component is fluorescein (FAM);
- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad;

wherein the method comprises the steps of

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) applying a RCA reaction mixture to the detection region of the LFD, wherein the RCA reaction mixture comprises
  - (1) a circular RCA template comprising the sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3';
  - (2) a mixture of dNTPs;
  - (3) a DNA polymerase, wherein the DNA polymerase is phi 29 DNA polymerase; and
  - (4) a detection component, wherein the detection component comprises a cyanine dye, such as SYBR Green II.
- (d) incubating the LFD;
- and

- (e) assessing the intensity on the test line.

[0026] Detection of insulin in a sample using a lateral flow assay device described herein may comprise assessing a test line to quantify a concentration of insulin in the sample using a portable device, including, but not limited to, a personal glucose monitor. In certain embodiments, the test line captures a component that increases in concentration as the insulin concentration increases in a sample.

[0027] In a third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer;
  - (ii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the glucose loaded liposome;
- and
- (iv) an absorbent pad.

[0028] In one embodiment related to the third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer;
  - (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the glucose loaded liposome;
- and
- (iv) an absorbent pad.

[0029] In one embodiment related to the third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
- (iii) a detection region comprising a test line comprising comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein the GLL-capture

molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3';

and;

(iv) an absorbent pad.

[0030] In embodiments related to the lateral flow assay device of the third aspect, provided is a method of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a sample comprising

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
- (iii) a detection region comprising a test line comprising comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein the GLL-capture molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3';

and;

(iv) an absorbent pad;

wherein the method comprises the steps of

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) isolating the test line comprised in the detection region of the LFD;
- (d) releasing the glucose from the GLL captured on the test line; and
- (e) assessing the glucose released from the GLL.

[0031] In embodiments related to the lateral flow assay device of the third aspect, the insulin probe may be conjugated to a magnetic bead using the interaction of biotin and streptavidin, wherein the aptamer comprised in the insulin probe is modified with biotin and the magnetic bead is modified with streptavidin.

## Definitions

[0032] Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising" and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

[0033] As used herein and in the appended claims, the singular form of “a”, “an”, and “the” may include the plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a biological sample” includes a plurality of such samples, and so forth. It is further noted that the claims may be drafted to exclude any optional element.

[0034] As used herein the term “about” can mean within 1 or more standard deviation per the practice in the art. Alternatively, “about” can mean a range of up to 20%, up to 10%, or up to 5%. In certain embodiments, “about” can mean to 5%. When particular values are provided in the specification and claims the meaning of “about” should be assumed to be within an acceptable error range for that particular value.

[0035] As used herein, the term “sample” includes, but is not limited to, a fluid, which may comprise insulin, a solution, which may comprise insulin, and a biological sample obtained from a human or animal subject. Biological samples include but are not limited to saliva, serum, blood, urine, or exhaled breath condensate. In certain embodiments, the sample may be fresh. It will be appreciated that a fresh sample includes, but is not limited to, a sample obtained from a subject and that is subjected to insulin detection by methods herein described within several second, for example, less than about 1 to about 3 minutes, after the sample is obtained. In related embodiments, a sample is directly applied to a sample region, wherein the sample is not pre-treated and/or purified prior to application to the sample region. In certain embodiments, the sample may be a stored sample. It will be appreciated that a stored sample may have been prepared and/or obtained from a subject and subjected to storage, for example in a refrigerator or freezer prior to subjecting the sample to insulin detection by methods herein described. In some embodiments, the sample may be phosphate buffered saline (PBS) spiked with different concentrations of insulin. In certain embodiment, a sample may be applied to a sample region wherein the sample is not subjected to any processing (for example, dilution, filtration, concentration) prior to application to the sample region. In certain embodiments, a sample may be concentrated prior to application to a sample region. In certain embodiments, a sample may be filtered prior to application to a sample region. In certain embodiments wherein the sample is blood, a lateral flow assay device may further comprise a sample filter membrane applied to the sample region.

[0036] As used herein, the term “aptamer” refers to an oligonucleotide or a peptide molecule that binds to a target molecule, for example insulin, with high specificity and high selectivity. In certain embodiments of the invention, suitable aptamers specific for insulin exhibit a dissociation constant ( $K_D$ ) of  $10^{-7}$  M or less, including  $10^{-8}$  M or less,  $10^{-9}$  M or less,  $10^{-10}$  M or less,  $10^{-11}$  M or less, or  $10^{-12}$  M. As used herein, “affinity” refers to the strength of binding, increased binding affinity being correlated with a lower dissociation constant ( $K_D$ ). In certain embodiments, the aptamer is an oligonucleotide. Oligonucleotide aptamers may be single-stranded DNA or RNA

molecules. Oligonucleotide aptamers may comprise about 20 to about 100, nucleic bases. In certain embodiments, an oligonucleotide aptamer comprises about 30 to about 50 nucleic bases. Aptamers suitable for the methods, devices, and kits herein described may be prepared by any known method, including synthetic, recombinant, and purification methods. In certain embodiments, the aptamer binds to insulin and is referred to herein as an “aptamer specific for insulin”. Certain embodiments of the methods, devices, and kits described herein comprise a molecule specific for an aptamer that may be comprised on the surface of a liposome. In embodiments related to an oligonucleotide aptamer, suitable molecules specific to an aptamer will be known to the skilled person and include, but are not limited to, an oligonucleotide that bind to an aptamer, wherein the affinity of the oligonucleotide for an aptamer is lower than the affinity of the aptamer for its specific target molecule.

[0037] As used herein, the term “insulin probe” refers to a molecule comprising at least one aptamer specific for insulin. In certain embodiments, an insulin probe comprises an aptamer specific for insulin, a control component, and a detection component. In certain embodiments, an insulin probe comprises an aptamer specific insulin, a target component, and a RCA capture sequence. In certain embodiments, an insulin probe comprises an aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the glucose loaded liposome (GLL) comprises a molecule specific for the aptamer.

[0038] In certain embodiments related to the first aspect, an insulin probe comprises an aptamer specific for insulin, a control component, and a detection component. As used herein, the term “control component” refers to a component that may be comprised in an insulin probe capable of capture (also referred to herein as binding) by a control line, wherein the control line comprises a control component binding molecule. It will be appreciated that a control component comprised in an insulin probe and a corresponding control component binding molecule are not particularly limited, and a control component may be considered to bind to a control component binding molecule and/or a control component binding molecule may be considered to bind to a control component. Suitable control component comprised in an insulin probe and corresponding control component binding molecule pairs include, but are not limited to, an oligonucleotide sequence comprising multiple adenine nucleotide residues (a poly A oligonucleotide sequence) and an oligonucleotide comprising multiple thymine nucleotide residues (a poly T oligonucleotide sequence). In certain embodiments, a control component comprises a poly A oligonucleotide sequence and a corresponding control component binding molecule comprises a poly T oligonucleotide sequence. In certain embodiments, a control component comprises a poly T oligonucleotide sequence and corresponding control component binding molecule comprises a poly A oligonucleotide sequence. In certain embodiments, a control component may comprise a molecule that is specifically bound by an antibody comprised in a corresponding control component binding molecule. For example, suitable control

components include fluorescein (FAM) and a corresponding control component binding molecule comprises an anti-FAM monoclonal antibody. In certain embodiments, the control component binding molecule may comprise biotin, wherein streptavidin is used such that the control component binding molecule may be immobilized on a control line in a detection region of a lateral flow assay device.

[0039] In certain embodiments of the kits, devices, and methods described herein a control component is comprised in a sample region or conjugate pad of a lateral flow assay device, wherein a corresponding control component binding molecule is comprised in a control line of a detection region of the lateral flow assay device. It will be appreciated that a control component comprised in a sample region or conjugate pad of a lateral flow assay device and a corresponding control component binding molecule are not particularly limited, and a control component may be considered to bind to a control component binding molecule and/or a control component binding molecule may be considered to bind a control component. In certain embodiments, a control component comprised in a sample or conjugate pad may comprise detection component. Suitable control component comprised in a sample region or conjugate pad and corresponding control component binding molecule pairs include, but are not limited to, biotin and streptavidin, and lectin and sugar. In certain embodiments, streptavidin may be conjugated to a detector component such as colloid metal (for example gold nanoparticles) or a fluorescent dye (such as Texas Red), wherein a corresponding control line comprises a control component binding molecule comprising biotin. In related embodiments, biotin may be immobilized to a control line with bovine serum albumin.

[0040] It will be appreciated in the methods, kits, and devices described herein wherein binding pairs of components are described, for example a target component and a corresponding target component binding molecule or a control component and a corresponding control component binding molecule, that suitable binding pairs may include, but are not limited to: antigen/antibody pairs, wherein antigen/antibody pairs may include, for example, but are not limited to natural epitope/antibody pairs (e.g., insulin epitope/anti-insulin), laboratory generated antigen/antibody pairs (e.g., digoxigenin (DIG)/anti-DIG; dinitrophenyl (DNP)/anti-DNP; dansyl-X/anti-dansyl; Fluorescein/anti-fluorescein; lucifer yellow/anti-lucifer yellow; rhodamine/anti-rhodamine, etc), peptide or polypeptide antigen/antibody pairs (e.g., FLAG, histidine tag, hemagglutinin (HA) tag, c-myc tag, glutathione S transferase (GST)), and the like.

[0041] As used herein, the term "detection component" refers to a component comprised in an insuline probe that provide a detectable and/or measurable signal. The skilled person will be familiar with particles including but not limited to polystyrene beads and magnetic nanoparticles that may be modified with a fluorescent dye or colorimetric detection component to produce a suitable detection component. In certain embodiments, the detection provides a colorimetric

signal or fluorescent signal. Suitable detection components providing a colorimetric signal are known in the art and include, but are not limited to, color dyes such as Nile Blue; colloidal metal particles such as colloidal gold (also referred to herein as gold nanoparticles (AuNPs)), colloidal silver (also referred to as silver nanoparticles), and the like; as well as carbon quantum dots, and the like. Methods to conjugate colloid metal (metal nanoparticles such as gold nanoparticles) to oligonucleotide are known in the art and include, but is not limited to methods comprising thiolated oligonucleotides. Suitable detection components providing a fluorescent signal are known in the art and include, but are not limited to fluorescent dye such as Texas Red (also known as sulforhodamine 101 acid chloride), fluorescein (FAM), tetramethylrhodamine (TMR), Carboxy tetramethyl-rhodamine (TAMRA), Carboxy-X-rhodamine (ROX), cyanine dyes, and the like; as well as quantum carbon dots, and the like. Methods to conjugate a fluorescent dye such as Texas Red to oligonucleotides are known in the art. Suitable oligonucleotides conjugated to a fluorescent dye may be obtained from commercial sources.

[0042] In certain embodiments related to the second aspect, the insulin probe comprises an aptamer specific for insulin, a target component, and a RCA capture sequence. As used herein, a "target component" refers to a component comprised in an insulin probe capable of capture (also referred to herein to as binding) by a test line comprising a target component binding molecule. It will be appreciated that a target component and a corresponding target component binding molecule are not particularly limited, and a target component may be considered to bind to a target component binding molecule and/or a target component binding molecule may be considered to bind a target component. In certain embodiments, a target component may comprise a molecule that is specifically bound by an antibody comprised in a corresponding target component binding molecule. For example, suitable target components include fluorescence dyes such as fluorescein (FAM) or fluorescein isothiocyanate (FITC) and a corresponding target component binding molecule comprising an anti-FAM monoclonal antibody or an anti-FITC monoclonal antibody, respectively. In certain embodiments, the anti-FAM or anti-FITC monoclonal antibody at a concentration of about 4mg/mL is coated on the surface of a detection region comprising a test line at a concentration of about 1 $\mu$ L/cm. In certain embodiments, a detection region is a nitrocellulose filter membrane. In certain embodiments, the target component binding molecule may comprise biotin, wherein streptavidin is used such that the target component binding molecule may be immobilized on a test line in a detection region of a lateral flow assay device. As used herein, "a RCA capture sequence" refers to a sequence binding a sequence in a circular Rolling Circle Amplification template. The sequence of a RCA capture sequence is not particularly limited so long as the RCA capture sequence enables binding to a circular RCA template and initiation of Rolling Circle Amplification.

[0043] In certain embodiments related to the third aspect, an insulin probe comprises a first aptamer specific for insulin bound to a liposome, wherein the liposome encapsulates glucose

(referred to herein as a glucose loaded liposome (GLL)), and wherein the surface of the liposome comprises a molecule specific for the first aptamer. In embodiments related to the third aspect, the concentration of glucose released from GLL captured on a test line correlates with the concentration of insulin in the sample. In embodiments related to a lateral flow assay device of the third aspect, a concentration of glucose released from a GLL captured on a test line may be measured by suitable methods that will be apparent to the skilled person including, but not limited to a glucose test strip, a personal glucose meter (GM), and the like.

[0044] In embodiments related to a lateral flow assay device of the third aspect, an insulin probe comprises an aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the glucose loaded liposome comprises a molecule specific for the aptamer. Suitable GLL-capture molecules will be apparent to the skilled person depending on the molecule comprised on the surface of a GLL. In certain embodiments, a GLL-capture molecule may be an insulin aptamer wherein the corresponding molecule on the surface of a GLL is an oligonucleotide specific for the insulin aptamer. In certain embodiments related to the third aspect, an aptamer comprised in the insulin probe (also referred to herein as a 'first aptamer') and an aptamer comprised in a GLL-capture molecule (which may also be referred to as a 'second aptamer') have the same sequence.

[0045] As used herein, the term "assess" or "assessing" and the like, in relation to a test line may refer to qualitative, semi-quantitative, or qualitative determination of a molecule captured on a test line. Semi-quantitative or qualitative assessment of a test line may be comprised in methods relating to detecting insulin in a sample above or below a certain threshold. Qualitative assessment of a test line may be comprised in methods relating to confirmation of the presence or absence of insulin in a sample. Semi-quantitative or qualitative assessment of a test line may comprise visual examination of a test line or visual examination of a test line in comparison to a known standard. Qualitative assessment of a test time may be comprised in methods wherein a concentration of insulin in a sample is determined. Suitable methods to quantitatively assess a test line of a lateral flow assay device will be apparent to the skilled person depending on the nature of the device, e.g. colorimetric or fluorescence quantification. The intensity of a test line may be quantified using a strip reader (e.g. Ax-2x Lateral Flow Reader, Axxin, Victoria, Australia). Qualitative assessment of a test line may comprise use of commercially available devices or a smartphone application.

[0046] As used herein, the term "immobilized" refers that a molecule (e.g., a target component binding molecule, a capture component binding molecule, molecule comprised in a test line or a control line of a detection region of a LFD herein described, etc.) maintain their position under the assay conditions. As such, an immobilized target component binding molecule and a test line of a detection region (or an immobilized control component binding molecule and

a control line of a detection region) may be non-covalently and stably associated with each other. Examples of non-covalent association include non-specific adsorption, binding based on electrostatic (e.g., ion-ion pair interactions), Van der Waals forces, hydrophobic interactions, hydrogen bonding interactions, streptavidin-biotin affinity interaction, and the like.

[0047] As used herein, the term “conjugated” refers to two molecules that are stably associated with each other. In certain embodiments conjugated molecules may be share a covalent attachment to one another.

### **Brief Description of the Figures**

[0048] Embodiments of the disclosure will now be described, by way of example only, with reference to the accompanying drawings as follows.

[0049] **Fig. 1:** Schematic overview of one embodiment related to the first aspect: the competitive assay-based LFD for the detection of insulin in a sample comprises of a sample region, a conjugation pad, detection region (nitrocellulose membrane) and an adsorbent pad assembled on a backing plate. An insulin probe comprising an aptamer specific for insulin, a control component (oligonucleotide sequence), and a detection component (AuNPs) is applied to the conjugation pad. A sample is applied to the sample region and laterally flows through the device via capillary action to the detection region. As the sample flows laterally along the device, the test line (comprising an oligonucleotide specific for the aptamer comprised in the insulin probe) captures the aptamer comprised in the insulin probe and the control line (comprising an oligonucleotide specific for the control component comprised in the insulin probe) captures the insulin probe. In the presence of insulin, insulin would combine with the insulin probe decreasing the insulin probe available for capture with the test line, causing the test line signal (from AuNPs comprised in the insulin probe) to weaken. In the presence or absence of insulin, the insulin probe is captured by the control line ensuring valid operation of the LFD. The intensity of the test line may be quantified using commercially available devices e.g. a strip reader (e.g. Ax-2x Lateral Flow Reader) or a smartphone application.

[0050] **Fig. 2:** Optimisation of the streptavidin proportion of the test line (T-line labelled as DNA1) for insulin (2A) and for control line (C-line labelled as DNA2) for control component (2B) in relation to colour intensity readout. DNA 1 and DNA 2 were immobilized on the detection region (nitrocellulose membrane) using streptavidin-biotin affinity reaction. Error bars are standard deviations (STDV), n=3 (LFD herein described in Example 1).

[0051] **Fig. 3:** Size distribution of gold nanoparticles (AuNPs) was measured using zeta sizer. The size of AuNPs for the competitive assay-based LFD described herein is 20 to 30nm. Error bars are standard deviations (STDV), n=3 (LFD herein described in Example 1).

[0052] **Fig. 4:** Optimisation of pH and AuNPs in relation to colour intensity readout. Insulin probe was conjugated to AuNPs and the effect of different pH (3, 6, 7, 9, 11 and 12 corresponding to strips from left to right, respectively) and concentrations of AuNPs (0, 0.008, 0.032, 0.056, 0.08, 0.16, 0.32  $\mu\text{M}$  corresponding to strips from left to right, respectively) was studied. The optimum pH for binding of aptamer to AuNPs was 7.0 (4A) and the optimum concentration of AuNPs was 0.08  $\mu\text{M}$  (4B). Error bars are standard deviations (STDV),  $n=3$  (LFD herein described in Example 1).

[0053] **Fig. 5:** Optimisation of NaCl salt concentration in buffer solution in relation to colour intensity readout. When AuNPs and insulin probe exist simultaneously in a solution, NaCl can help to form a stable Au-S bond between the thiol group tagged to the aptamer comprised in the insulin probe and the AuNPs to increase the load capacity. To investigate the influence of NaCl, different concentrations of NaCl were added to the solution of AuNPs and insulin probe comprising aptamers. With the increase of NaCl concentration from 0 to 100  $\mu\text{M}$ , the intensity decreased, signifying that more insulin probe attached to the surface of the AuNPs. When the concentration of NaCl reached 80  $\mu\text{M}$ , the load capacity of insulin probe was almost saturated. Thus, 80  $\mu\text{M}$  of NaCl is the optimum concentration and was used for detection. Error bars are standard deviations (STDV),  $n=3$  (LFD herein described in Example 1).

[0054] **Fig. 6:** Calibration curve for the detection of insulin in PBS (6A) and representative results (6B). Competitive assay-based LFD can be used for detection of insulin with the linear range of 0.01 ng/ mL to 150 ng/ mL (6A) and the detection limit was 0.01 ng/ mL which is within the detection limit of insulin in medical research (0.35 ng/ mL). The initial results of the colorimetric competitive assay-based LFD for the detection of insulin in PBS is illustrated 6B. The OD (optical density) is inversely proportional to the colour intensity on the test line ( $1/I_{\text{t-line}}$ ). Error bars are standard deviations (STDV),  $n=3$  (LFD herein described in Example 1).

[0055] **Fig. 7:** Evaluation of the Competitive assay-based LFD performance against ELISA. The performance of the competitive assay-based LFD for the detection of insulin in buffer with the concentration of 1, 2, 5, 10, 20 ng/mL was evaluated using the commercially available human insulin ELISA kit. The competitive assay-based LFD has comparable performance to the ELISA with similar trend when the insulin concentration increases. The OD (optical density) is inversely proportional to the color intensity on the test line ( $1/I_{\text{t-line}}$ ). Error bars are standard deviations (STDV),  $n=3$  (LFD herein described in Example 1).

[0056] **Fig. 8:** Insulin detection in saliva spiked with 0.03 ng/mL, 0.3 ng/mL and 1 ng/mL of insulin. 50  $\mu\text{L}$  of saliva was added to the sample region of each LFD. The T-line and C-line were visible in 6 minutes. The strip on the right side represent saliva spiked with 1 ng/mL, the strip in the middle represent saliva spiked with 0.3 ng/mL and the strip on the left side represent saliva spiked with 0.03 ng/mL (LFD herein described in Example 1).

[0057] **Fig. 9:** Insulin detection in blood based on competitive assay-based LFD. Blood samples were diluted 100 times and spiked with 0.01 ng/mL, 0.02 ng/mL, 0.04 ng/mL, 0.2 ng/mL and 1 ng/mL of insulin (LFD herein described in Example 1).

[0058] **Fig. 10:** Optical signal changes with the concentration of insulin (10A). The calibration curve for detection of insulin by fluorescence (Texas Red) is shown in 10B. The competitive assay-based LFD can be used for the detection of insulin by fluorescence readout with a linear range of about 0.01 ng/ mL to about 100 ng/ mL and the detection limit was about 0.01 ng/ mL which is within the detection limit of insulin in medical research (0.35 ng/ mL) (10B). The performance of the competitive assay-based LFD was evaluated using different concentrations of insulin (0.5, 1, 2, 4, 8 ng/ mL) and comparing the results to the commercially available human insulin ELISA kit. The performance of the competitive assay-based LFD was comparable to ELISA (10C). The initial results with representative strips are shown in 10D. The OD (optical density) is inversely proportional to the colour intensity on the test line ( $1/I_{t\text{-line}}$ ) (LFD herein described in Example 1).

[0059] **Fig. 11:** Schematic overview of one embodiment of the disclosure: Rolling Circle Amplification (RCA)-based LFD relating to the second aspect. The insulin probe absorbed onto Graphene Oxide (GO) are comprised in a conjugation pad of a lateral flow assay device (LFD). The insulin probe comprises an aptamer specific for insulin, a target component (FAM), and a capture sequence). A control component (Streptavidin-AuNPs) is comprised in a conjugate pad. A target component binding molecule (anti-FAM monoclonal antibody) is pre-immobilized on a test line comprised in a detection region of a LFD. Biotin modified with bovine serum albumin (BSA) is pre-immobilized on a control line comprised in a detection region of a LFD. A sample is applied to the sample region and flows to the conjugation pad and to the detection region. In the presence of insulin, the insulin probe will desorb from the GO and is captured by the target component binding molecule comprised in the test line. The LFD may be incubated for about 10 minutes. After incubation, an RCA reaction mixture is applied to the detection region and RCA is triggered.

[0060] **Fig. 12:** Schematic overview of one embodiment of the disclosure: liposome signal amplification-based LFD relating to the third aspect. The liposome signal amplification-based LFD may use a glucose strip and glucose meter (GM) for the detection of insulin. Insulin probe bound to GLL is comprised in a conjugate pad of a LFD. A GLL-capture molecule specific for the molecule comprised on the surface of the GLL is adsorbed on to a test line comprised in a detection region of a LFD. A sample is applied to a sample region and laterally flows to the LFD. In the presence of insulin, the GLL is released from the insulin probe and is available for capture by the GLL-capture molecule comprised in the test line. The captured GLL is isolated by isolation of the test line. The glucose is then released from the liposome by treating with non-ionic

surfactant. A sample of the released glucose may be applied to a glucose strip and glucose measured using a personal GM. The concentration of glucose measured is related to the concentration of insulin in the sample applied to the LFD.

[0061] **Fig. 13:** Suitability of an LFD described herein for detection of insulin. (A) Color intensity on the test line ("t-line") after adding insulin (3 ng/mL), glucose (1 mg/mL), uric acid (0.1 mg/mL), human serum albumin (HAS, 50 mg/mL), or IgG (20 mg/mL) to an embodiment of an LFD herein described. (B) Color intensity on the t-line after adding glucose (1 mg/mL), uric acid (0.1 mg/mL), human serum albumin (HAS, 50 mg/mL), or IgG (20 mg/mL), in the presence of insulin (3 ng/mL) to an embodiment of an LFD herein described. It was observed (13 A) that the color intensity was weaker for insulin than that for other molecules present in human blood, which may interfere with the detection of insulin from blood sample, blood suggesting that the LFD herein described is specific to insulin. The specificity for insulin was confirmed by detecting insulin in the presence of an interference molecule with an LFD herein described, where a color intensity decrease was observed (13 B), which confirms that an LFD herein described was selective for insulin (LFD herein described in Example 1).

[0062] **Fig. 14:** Calibration curve for the detection of insulin in phosphate buffered-saline (PBS) using an LFD as herein described based on RCA amplification. RCA based LFD can be used for detection of insulin with a linear range of 0.001 ng/mL to 50 ng/mL and the detection limit is 0.001 ng/mL. Error bars are standard deviations (STDV), n=3.

[0063] **Fig. 15:** Optimization of insulin binding aptamer concentration for colorimetric LFD; AuNPs concentration (0.08  $\mu$ M); AuNPs pH (7.0), DNA 1 and DNA 2 (75  $\mu$ M), streptavidin (1 mg/mL). The probe concentration ranged from 1, 10, 20, 30, 40 and 50  $\mu$ M. The intensity saturated after 30  $\mu$ M as most of the probe had bonded to T and C-lines. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0064] **Fig. 16:** Optimization of insulin binding aptamer concentration for fluorescent based LFD. The probe concentration ranged from 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10  $\mu$ M. The intensity saturated after 8  $\mu$ M. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0065] **Fig. 17:** Optimization of DNA 1 and DNA 2 at T-line and C-line for colorimetric LFD. Different concentrations (0, 25, 50, 75, 100 and 125  $\mu$ M) were tested with insulin concentration of 3ng/mL. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0066] **Fig. 18:** Optimization of DNA 1 and DNA 2 at T-line and C-line for fluorescent based LFD. Different concentrations (0, 2, 4, 6, 8 and 10  $\mu$ M) were tested with insulin concentration of 3ng/mL. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0067] **Fig. 19:** Optimization of incubation time for fluorescent based LFD. Incubation times tested were 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes. It was observed that 25 minutes was the optimum incubation time. Error bars are standard deviations (STVD),  $n=3$  (LFD herein described in Example 2).

[0068] **Fig. 20:** Calibration curve for insulin for the colorimetric LFD. Different insulin concentrations (0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 30 and 50 ng/mL) (70  $\mu$ L) were added to test strips and incubated for 12 minutes. It was observed that the relative intensity (T/C) decreased with increasing concentration of insulin ( $R^2 = 0.9597$ ). The detection for each concentration was repeated 10 times. The limit of detection (LOD) was determined to be 0.01ng/mL. Error bars are standard deviations (STVD),  $n=10$  (LFD herein described in Example 2).

[0069] **Fig. 21:** Calibration curve for insulin for fluorescent based LFD. Different insulin concentrations (0, 0.03, 0.1, 0.5, 1, 3, 5, 10, 30 and 100ng/mL) (70  $\mu$ L) were added to test strips and incubated for 25 minutes. It was observed that the relative intensity (T/C) decreased with increasing concentration of insulin ( $R^2 = 0.9961$ ). The detection for each concentration was repeated 10 times. The limit of detection (LOD) was determined to be 0.01ng/mL. Error bars are standard deviations (STVD),  $n=10$  (LFD herein described in Example 2).

[0070] **Fig. 22:** (A) Specificity of colorimetric LFD for insulin (0.3ng/mL) against interfering agents: IgG, uric acid, glucose, BSA and HAS (300 ng/mL). (B) Selectivity of colorimetric LFD for insulin (0.3 ng/mL) against interfering agents: IgG, uric acid, glucose, BSA and HAS (300 ng/mL). (C) Stability of colorimetric LFD from 0, 5, 10, 15, 20, 25 and 30 days; insulin concentration (0.5 ng/mL). Error bars are standard deviations (STVD),  $n=3$  (LFD herein described in Example 2).

[0071] **Fig. 23:** (A) Specificity of fluorescent based LFD for insulin (0.3ng/mL) against interfering agents: IgG, uric acid, glucose, BSA and HAS (300 ng/mL). (B) Selectivity of fluorescent based LFD for insulin (0.3 ng/mL) against interfering agents: IgG, uric acid, glucose, BSA and HAS (300 ng/mL). (C) Stability of the fluorescent based LFD from 0, 5, 10, 15, 20, 25 and 30 days; insulin concentration (0.5 ng/mL). Error bars are standard deviations (STVD),  $n=3$  (LFD herein described in Example 2).

[0072] **Fig. 24:** (A) General trend of insulin levels in saliva at different time intervals of the day. (B) Detection of insulin levels in saliva samples of healthy human subjects using the colorimetric LFD. The saliva samples (70 $\mu$ L) were collected (in commercially available saliva collection tube) at different intervals of the day, i.e. fasting (over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm). Each bar in Fig. 24B represent from the left hand side to the right hand side: fasting, breakfast, lunch and dinner, respectively. Insulin levels were determined on

the LFD at about 30 minutes after each meal. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0073] **Fig. 25:** Detection of insulin levels in blood samples of healthy human subjects using the colorimetric LFD. The blood samples (a drop of blood collected from a finger prick) were collected at different intervals of the day, i.e. fasting (over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm). Insulin levels were determined on the LFD at about 30 minutes after each meal. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0074] **Fig 26:** Detection of insulin levels in (A) saliva and (B) blood samples of healthy human subjects using the fluorescent based LFD. The saliva (70  $\mu$ L, collected in commercially available saliva collection tube) and blood samples (a drop of blood collected from a finger prick) were collected at different intervals of the day, i.e. fasting (over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm). Insulin levels were determined on the LFD at about 30 minutes after each meal. Error bars are standard deviations (STVD), n=3 (LFD herein described in Example 2).

[0075] **Fig. 27:** Calibration curve for insulin using the human insulin ELISA kit. The ELISA readings were taken at 450nm. The optical density increases linearly with increasing insulin concentration ranging from 0.1 to 1 ng/mL; linear coefficient of 0.9869. The limit of detection was 0.1 ng/mL. Error bars are standard deviations (STVD), n=3 (Example 2).

[0076] **Fig. 28:** Validation of the colorimetric LFD herein described in Example 2 against the human insulin ELISA kit in (A) PBS, (B) serum, (C) saliva, (D) blood. Different concentrations of insulin (0.1, 0.3, 0.5, 0.7 and 1 ng/mL) were spiked into PBS, serum, saliva and blood samples and insulin levels were measured using ELISA and the LFD herein described in Example 2. The correlation between the 2 detection methods was about 88-90%. Error bars are standard deviations (STVD), n=3.

[0077] **Fig. 29:** Validation of the fluorescent LFD herein described in Example 2 against the human insulin ELISA kit in (A) PBS, (B) serum, (C) saliva, (D) blood. Different concentrations of insulin (0.1, 0.3, 0.5, 0.7 and 1 ng/mL) were spiked into PBS, serum, saliva and blood samples and insulin levels were measured using ELISA and the LFD herein described in Example 2. Error bars are standard deviations (STVD), n=3.

[0078] **Fig 30:** The Smartphone based application works with a 3D-printed black box herein described in Example 2. The function of the black box is to eliminate the effect of light from the surrounding environment. The LFD test strip is to be inserted into the notch inside the black box and the smartphone is to be inserted into the notch located on top of the box.

[0079] **Fig. 31:** Comparison of data collected using the Smartphone App (represented by the bar on the right hand side) and the Axxin reader (represented by the bar on the left hand side). Different insulin concentrations (0.01, 0.05, 0.1, 0.5 and 1ng/mL) were spiked into PBS buffer and insulin levels were measured using the Ax-2x lateral flow read (Axxin reader) and the Smartphone App. The reading methods had a correlation of about 75%. Error bars are standard deviations (STVD), n=5.

#### Detailed description of embodiments

[0080] Methods described herein relate to detecting insulin comprising applying a sample to a lateral flow assay device. As a "lateral flow" assay device, the device is configured to receive a sample at a sample region and to provide for the sample to move laterally, via, e.g. wicking, by capillary action from the sample region to a detection region. In certain embodiments, the lateral flow assay device further comprises one or more conjugation pad(s), wherein the lateral flow assay device is configured to provide for lateral flow of a sample from a sample region to one or more conjugation pad(s) prior to reaching a detection region. In related embodiments of a lateral flow assay device, a sample region is in contact with a conjugation pad and the conjugation pad is in contact with one end of a detection region such that the lateral flow assay device is configured to allow a sample to flow from the sample region, to a conjugation pad and finally to a detection region. In related embodiments of a lateral flow assay device, a sample region is in contact with a first conjugation pad, the first conjugation pad is in contact with a second conjugation pad, and the second conjugation pad is in contact with one end of a detection region such that the lateral flow assay device is configured to allow a sample to flow from the sample region, to a first conjugation pad, followed by a second conjugation pad, and finally to a detection region. In certain embodiments of the lateral flow assay device, the device further comprises an absorbent pad in contact with a detection region such that the device is configured to allow the flow of a sample from a sample region to a detection region and finally to the absorbent pad.

[0081] In embodiments related to a lateral flow assay device of the first, second, or third aspect, a lateral flow assay further comprises a backing plate. It will be appreciated that backing plates for a lateral flow assay device described herein are not particularly limited and any suitable material that does not substantially absorb liquid may be applied to a LFD as herein described. Suitable materials for a backing plate of a lateral flow assay device herein described include hydrophobic, non-porous materials including but not limited to polystyrene, polyethylene, or polypropylene. For example, in certain embodiments, the LFD comprises a nitrocellulose backed membrane. Suitable commercially available materials will be known to the skilled person.

[0082] Suitable materials for a sample region, conjugation pad, or a detection region that may be comprised in a lateral flow assay device described herein include, but are not limited to organic or inorganic polymers, and natural and synthetic polymers, including glass fiber, cellulose,

nylon, cross-linked dextran, various chromatographic papers and nitrocellulose. It will be appreciated that suitable materials will enable a sample to flow laterally, via capillary action, along a LFD herein described. In certain embodiments of the lateral flow assay device related to the first, second, or third aspect, the detection region is a nitrocellulose membrane. In certain embodiments, a sample region and a conjugation pad may be composed of the same material. In certain embodiments, a lateral flow assay device comprises a sample region in capillary contact with a detection region. In related embodiments, insulin probe and/or control components may be comprised in a sample region. Suitable commercially available materials will be known to the skilled person. Commercially available materials may be used for a sample region, conjugation pad, and/or detection region that may be comprised in a lateral flow assay device herein described.

[0083] Embodiments of the methods, kits, and lateral flow assay devices of the first, second, or third aspect, the lateral flow assay device may further comprise a sample filter membrane applied to the sample region. The sample filter membrane may be composed of any suitable material including, but not limited to, a hydrophobic material capable of filtering out cells (for example blood cells) from fluids. In certain embodiments, a commercially available membrane, such as a Vivid Plasma Separation Membrane or a membrane similar thereto, may be used in a LFD herein described. Suitable sample membranes may have a filter size of about 0.22  $\mu\text{m}$  to about 10  $\mu\text{m}$ . In certain embodiments, the sample filter membrane has a filter size of less than about 10  $\mu\text{m}$ , less than about 5  $\mu\text{m}$ , or less than about 1  $\mu\text{m}$ . In certain embodiments, the sample filter membrane has a size of about 0.5  $\mu\text{m}$ . In certain embodiments, the sample filter membrane has a size of about 0.25  $\mu\text{m}$  or less.

[0084] Suitable materials for an absorbent pad include, but are not limited to hydrophilic materials such as cellulose and porous polymers. Commercially available materials may be used for an absorbent pad that may be comprised in a lateral flow assay device herein described.

[0085] In embodiments related to the first aspect, provided is a lateral flow assay device (LFD) comprising a competitive assay for detection of insulin from a sample. Embodiments related to the first aspect comprise use of an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component. Embodiments related to the first aspect comprise a competitive assay wherein insulin and a molecule comprised on a test line of a lateral flow assay device compete for an insulin probe. In embodiments related to the first aspect, an insulin probe comprises a detection component and thereby an insulin probe captured on a test line of a LFD is detectable. In embodiments related to the first aspect, a detection region of a lateral flow assay device (LFD) is configured such that a sample flows past a test line before a control line.

[0086] In an embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component; and
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe.

[0087] In an embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component;
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe;

and;

- (iv) an absorbent pad.

[0088] In methods relating to detecting insulin using a lateral flow assay device (LFD) of the first aspect, a sample is applied to the sample region of a LFD and LFD is incubated. Incubation comprises allowing a LFD to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from the sample region to the detection region. In embodiments further comprising a conjugation pad, incubation comprises allowing a LFD to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from the sample region to the conjugation pad followed by the detection region. In methods relating to the first aspect, the sample and insulin probe laterally flow to a detection region. In the presence of insulin, a molecule specific for an aptamer comprised in an insulin probe, binding molecule comprised on a test line is in competition with insulin for binding to an insulin probe. In methods related to the first aspect, an insulin probe has higher affinity to insulin than the affinity of the insulin probe to the molecule specific for the aptamer comprised on a test line. Thereby, as insulin concentration increases, the concentration of insulin probe available for capture by a test line of an LFD decreases.

[0089] In one embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprising an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise gold nanoparticles (AuNPs);
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprising a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
    - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';
- and
- (iv) an absorbent pad.

[0090] In one embodiment related to the first aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and and wherein the detection component comprise the fluorescence dye Texas red;
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprising a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
    - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';
- and
- (iv) an absorbent pad.

[0091] In an embodiment related to the lateral flow assay device of the first aspect, provided is a method of detecting insulin comprising a lateral flow assay device (LFD) comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprising an aptamer specific for insulin, a control component, and a detection component;
- (iii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe;

and

- (iv) an absorbent pad;

wherein said method comprises:

- (a) applying a sample to the sample region;
- (b) incubating the LFD;
- (c) qualifying the intensity of the test line comprised in the detection region.

[0092] In an embodiment related to the lateral flow assay device of the first aspect, provided is a method of detecting insulin comprising a lateral flow assay device (LFD) comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise the fluorescence dye Texas red;
- (iii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprising a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';

and

- (iv) an absorbent pad;

wherein said method comprises:

- (a) applying a sample to the sample region;
- (b) incubating the LFD;
- (c) qualifying the intensity of the test line comprised in the detection region.

[0093] In an embodiment related to the lateral flow assay device of the first aspect, provided is a method of detecting insulin comprising a lateral flow assay device (LFD) comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise gold nanoparticles (AuNPs);
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprising a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3';

and

- (iv) an absorbent pad;

wherein said method comprises:

- (a) applying a sample to the sample region;
- (b) incubating the LFD;
- (c) qualifying the intensity of the test line comprised in the detection region.

[0094] In embodiments related to methods, kits, and lateral flow assay devices of the first aspect, the lateral flow assay device (LFD) is incubated after applying a sample to the sample region for about 2 minutes to about 20 minutes, about 2 minutes to about 15 minutes, or about 2 minutes to about 10 minutes. In certain embodiments, the LFD is incubated for about 10 minutes to about 15 minutes.

[0095] In embodiments related to methods, kits, and lateral flow assay devices of the first aspect, the method has an insulin detection limit of about 0.01 ng/mL. In certain embodiments related to methods, kits, and lateral flow assay devices of the first aspect, the method has a linear insulin detection range of about 0.01 ng/mL to about 150 ng/mL. In certain embodiments related to methods, kits, and lateral flow assay devices of the first aspect, the method has a linear insulin detection range of about 0.01 ng/mL to about 100 ng/mL. It will be appreciated that insulin concentrations in blood have been reported in the range of about 0.1 ng/ml to 5 ng/ml, depending on whether the subject is fasting or non-fasting when the sample is obtained. It has been reported that the concentration of insulin in saliva is about 10% of that measured in serum (B. Fabre et al., Endocrine Connections, 2012, 1, 58-61).

[0096] In embodiments related to methods, kits, and lateral flow assay devices of the first aspect, the method comprises detecting at least about 85% of insulin in a sample. In certain embodiments, the method comprises detecting at least about 90% of insulin in a sample. In certain embodiment, the method comprises detecting at least about 95% of insulin in a sample.

[0097] In embodiments related to a lateral flow assay device of the first aspect, the control component binding molecule may be immobilized to the control line comprised in a detection region of the LFD using a streptavidin-biotin affinity reaction. In certain embodiments, the control component binding molecule comprises biotin, wherein streptavidin is used to immobilize the control component binding molecule to a control line. In certain embodiments, the molecule specific for an aptamer comprised in the insulin probe may be immobilized to the test line comprised in a detection region of the LFD using a streptavidin-biotin affinity reaction. In certain embodiments, the molecule specific for an aptamer comprised in the insulin probe comprises biotin, wherein streptavidin is used to immobilize the molecule to a control line.

[0098] In embodiments related to a lateral flow assay device of the first aspect, the ratio of molecule specific for the aptamer comprised in an insulin probe, which is immobilized on a test line, to control component binding molecule, which is immobilized on a control line, is 1:3.

[0099] Embodiments related to methods, kits, and lateral flow assay devices of the first aspect, may further comprise inspection of the signal of the control line to confirm valid operation of the lateral flow assay device. Inspection may comprise visual confirmation of signal on the control line.

[00100] Kits relating to a lateral flow assay device of the first aspect are disclosed. Kits may comprise a lateral flow assay device wherein an insulin probe is provided for applying to a sample pad or conjugation pad of a LFD.

[00101] In embodiments related to the second aspect, provided is a lateral flow assay device (LFD) comprising Rolling Circle Amplification (RCA) based detection of insulin from a sample. Embodiments related to the second aspect comprise use of an insulin probe comprising an aptamer specific for insulin, a target component, and a RCA capture sequence. Embodiments related to the second aspect comprise an insulin probe absorbed onto graphene oxide (GO). In embodiments related to the second aspect, in the presence of insulin, an insulin probe desorbs from graphene oxide and is available for capture by a test line comprised in a detection region of a LFD. In embodiments related to the second aspect, a detection region of a lateral flow assay device (LFD) is configured such that a sample flows past a test line before a control line. Embodiments related to the second aspect comprise a test line comprised in a detection region of a LFD capturing a component that increases in concentration as the insulin concentration increases in a sample; thereby the concentration of insulin in a sample may be assessed.

[00102] In an embodiment related to the second aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising:
  - (a) an insulin probe comprising an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); and
  - (b) a control component;

and

- (ii) a detection region comprising
  - (a) a control line comprising a control component binding molecule; and
  - (b) a test line comprising a target component binding molecule.

[00103] In an embodiment related to the second aspect, provided is a lateral flow assay device for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO);
- (ii) a conjugation pad comprising a control component;
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule; and
  - (b) a test line comprising a target component binding molecule;

and;

- (iv) an absorbent pad.

[00104] In methods relating to detecting insulin using a lateral flow assay device related to the second aspect, the method comprises applying a sample to the sample region and incubating the lateral flow device. Incubation comprises allowing a lateral flow assay device to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from the sample region to the detection region. In embodiments comprising a conjugation pad, incubation comprises allowing a lateral flow assay device to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from the sample region to the conjugation pad followed by the detection region. In methods relating to the second aspect, the sample, insulin probe, and control component laterally flow to a detection region. In the presence of insulin, an insulin probe comprising an aptamer specific for insulin, a target component, and an RCA capture sequence, desorbs from graphene oxide and is available for capture by a target component binding molecule comprised on the test line. Methods related to the lateral flow assay device of the second aspect, further comprise applying a Rolling Circle Amplification (RCA) reaction mixture to the detection region of an LFD. In certain embodiments, a RCA reaction mixture comprises a circular RCA template, a mix of deoxynucleotides (dNTPs),

a DNA polymerase, and a detection component. In certain embodiments, a RCA reaction mixture further comprises pullulan. It will be understood that a mix of dNTPs comprises a mixture of four nucleotides (dATP, dCTP, dGTP, dTTP) (2'-deoxynucleoside 5'-triphosphates). In one embodiment, a RCA template encodes a functional nucleic acid sequence such as DNAzyme, and a RCA reaction mixture further comprises 3,3',5,5'-tetramethylbenzidine (TMB) (as a detection component) and hemin. In one embodiment, a detection component comprises a cyanine dye, including but not limited to commercially available cyanine dyes such as SYBR Green II, SYBR Gold, SYBR Green I, Picogreen, Oligreen, and the like. In methods related to a lateral flow assay device of the second aspect, a control component comprised in a sample pad or a conjugation pad laterally flows to a detection region wherein the control component is available for capture by a control component binding molecule comprised in a control line. In certain embodiments related to the second aspect, a control component comprises a detection component, wherein the detection component provides for verification, including visual verification, of the operation of the lateral flow assay device. Suitable detection component comprised in a control component related to the second aspect are not particularly limited and may include, but are not limited to, gold nanoparticle (AuNPs), an antibody or an aptamer. In certain embodiments related to the second aspect, a control component comprises streptavidin and a control component binding molecule comprises biotin. In embodiments related to the second aspect, an insulin probe comprises a capture sequence designed to hybridize to a circular RCA template. In embodiments related to the second aspect, insulin captured by an insulin probe, which has been desorbed from GO, is available for capture by a target binding molecule comprised in a test line. As an insulin probe captured on a test line comprises an RCA capture sequence, applying a RCA reaction mixture initiates RCA.

[00105] Rolling Circle Amplification (RCA) is known in the art and the skilled person will be familiar with suitable concentrations of ingredients and conditions for carrying out RCA. (See, eg, Ying et al., *Talanta*, 2017, 164, 432-438). Suitable DNA polymerases include, but are not limited to, phi 29 polymerase or Bst large fragment polymerase. In certain embodiments, the DNA polymerase is phi 29 polymerase. Suitable incubation conditions for RCA are known to the skilled person and include, but are not limited to, incubating a lateral flow device at about 20°C to about 35°C, or at room temperature (eg about 20°C to about 25°C), for about 15 minutes to about 30 minutes.

[00106] In one embodiment related to a lateral flow assay device of the second aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe

comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component is FAM;

- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad.

[00107] In embodiments related to the lateral flow assay device of the second aspect, provided are methods of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component is fluorescein (FAM);
- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising:
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad;

wherein the method comprises the steps of:

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) applying a RCA reaction mixture to the detection region of the LFD, wherein the RCA reaction mixture comprises
  - (1) a circular RCA template comprising the sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3';

- (2) a mixture of dNTPs;
- (3) a DNA polymerase, wherein the DNA polymerase is phi 29 DNA polymerase;
- (4) a detection component, wherein the detection component is 3,3',5,5'-tetramethylbenzidine (TMB); and
- (5) hemin;
- (d) incubating the LFD;
- and
- (e) assessing the intensity on the test line.

[00108] In embodiments related to the lateral flow assay device of the second aspect, provided is a method of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a biological sample comprising

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and an RCA capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO); wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component is fluorescein (FAM);
- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs);
- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
  - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody;

and;

- (iv) an absorbent pad;

wherein the method comprises the steps of

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) applying a RCA reaction mixture to the detection region of the LFD, wherein the RCA reaction mixture comprises
  - (1) a circular RCA template comprising the sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3';
  - (2) a mixture of dNTPs;
  - (3) a DNA polymerase, wherein the DNA polymerase is phi 29 DNA polymerase; and
  - (4) a detection component, wherein the detection component comprises a cyanine dye, such as SYBR Green II.

- (d) incubating the LFD;
- and
- (e) assessing the intensity on the test line.

[00109] In certain embodiments related to methods, kits, and lateral flow assay devices of the second aspect, the lateral flow assay device (LFD) is incubated after applying a sample to the sample region, and prior to addition of a RCA mixture, for about 2 minutes to about 20 minutes, about 2 minutes to about 15 minutes, or about 2 minutes to about 10 minutes. In certain embodiments, a LFD is incubated after applying a sample to the sample region, and prior to addition of a RCA mixture, for about 10 minutes to about 15 minutes.

[00110] In certain embodiments related to methods, kits, and lateral flow assay devices of the second aspect, the lateral flow assay device (LFD) is incubated after applying a sample to the sample region and after applying a RCA reaction mixture for about 15 minutes to about 30 minutes.

[00111] In certain embodiments related to methods, kits, and lateral flow assay devices of the second aspect, a RCA reaction mixture may be applied to a sample pad of a LFD simultaneously with, prior to, or following applying a sample to a sample pad, wherein after applying a RCA mixture and a sample, the LFD is incubated at about 20°C to about 35°C, or at room temperature (eg about 20°C to about 25°C), for about 15 minutes to about 30 minutes.

[00112] In embodiments related to methods, kits, and lateral flow assay devices of the second aspect, the method has an insulin detection limit of about 0.001 ng/ mL. In embodiments related to methods, kits, and lateral flow assay devices of the second aspect, the method has a linear insulin detection range of about 0.001 ng/mL to about 50 ng/mL.

[00113] In embodiments related to methods, kits, and lateral flow assay devices of the second aspect, the method comprises detecting at least about 85% of insulin in a sample. In certain embodiments, the method comprises detecting at least about 90% of insulin in a sample. In certain embodiment, the method comprises detecting at least about 95% of insulin in a sample.

[00114] In embodiments related to a lateral flow assay device of the second aspect, a control component binding molecule may be immobilized to a control line comprised in a detection region of a LFD using non-specific binding affinity associated with bovine serum albumin. In certain embodiments, a control component binding molecule comprises biotin conjugated to bovine serum albumin. In embodiments related to a lateral flow assay device of the second aspect, a target component binding molecule may be directly immobilized to a test line comprised in a detection region of a LFD by exposing the target component binding molecule to a test line of the LFD.

[00115] Embodiments related to methods, kits, and lateral flow assay devices of the second aspect, may further comprise inspection of the signal of a control line to confirm valid operation of the lateral flow assay device. Inspection may comprise visual confirmation of signal on a control line.

[00116] Kits relating to a lateral flow assay device of the second aspect are disclosed. Kits may comprise a lateral flow assay device wherein an insulin probe absorbed onto graphene oxide can be provided for applying to a sample pad or a conjugation pad of a LFD and/or a control component is provided for applying to a sample pad or conjugation pad. In certain embodiments relating to kits of the second aspect, an insulin probe and graphene oxide are provided wherein the insulin probe may be absorbed onto graphene oxide prior to applying the insulin probe absorbed onto graphene to a sample pad or conjugation pad of a LFD. Kits may further comprise a RCA reaction mixture supplied in as one or more components for preparation prior to application to a LFD.

[00117] In embodiments related to the third aspect, provided is a lateral flow assay device (LFD) comprising liposome encapsulating glucose (referred to herein as a glucose loaded liposome (GLL), wherein a concentration of insulin in a sample corresponds to the concentration of glucose released from GLL. Embodiments related to the third aspect comprise use of an insulin probe comprising an aptamer specific for insulin bound to a liposome encapsulating glucose (GLL), wherein the surface of the GLL comprises a molecule specific for the aptamer comprised in the insulin probe. Embodiments related to the third aspect comprise a competition for an insulin probe between insulin and a molecule comprised on the surface of the GLL. In methods of detecting insulin related to the third aspect, in the presence of insulin, an insulin probe binds insulin and releases the GLL and thereby the GLL is available for capture by the GLL-capture molecule comprised in a test line of the lateral flow assay device. Embodiments related to the third aspect comprise a test line comprised in a detection region of a LFD, wherein an increase in concentration of insulin in a sample corresponds to an increase in GLL released from an insulin probe available for capture on a test line. Detection of glucose by, eg, a glucose meter (GM), thereby corresponds to the insulin concentration of a sample.

[00118] Detection of insulin in a sample using a lateral flow assay device related to the third aspect, as describe herein, may comprise assessing a test line to quantify a concentration of insulin in the sample using a portable device, including, but not limited to, a personal glucose monitor.

[00119] In embodiments related to the third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer; and
- (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of GLL.

[00120] In embodiments related to the third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a sample comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer;
- (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL;

and

- (iv) an absorbent pad.

[00121] In methods relating to detecting insulin using a lateral flow assay device of the third aspect, the method comprises applying a sample to a sample region and incubating a lateral flow device. Incubation comprises allowing a lateral flow assay device to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from a sample region to the detection region. In embodiments comprising a conjugation pad, incubation comprises allowing a lateral flow assay device to remain at a temperature, for example room temperature (eg about 20°C to about 25°C), such that the sample flows from a sample region to the conjugation pad followed by the detection region. In methods relating to the third aspect, the sample and insulin probe laterally flow to a detection region. In methods relating to a lateral flow assay device of the third aspect, an insulin probe comprises an aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the aptamer comprised in the insulin probe, and wherein the insulin probe is bound to the GLL via the interaction of the aptamer with the molecule on the surface of the GLL. In certain embodiments, the molecule comprised on the surface of a GLL may be a nucleotide sequence specific for an aptamer comprised in an insulin probe. In certain embodiments, the molecule comprised on the surface of a GLL is an antibody specific for an aptamer comprised in the insulin probe. In embodiments related to a lateral flow assay device of the third aspect, insulin completes for binding to an insulin probe with a molecule comprised on the surface of a GLL, wherein the affinity of the aptamer comprised in the insulin probe is higher for insulin than for the molecule comprised on the surface of a GLL. Thereby, in the presence of insulin, the GLL is unbound or released from the insulin probe and is available for capture by the GLL-capture molecule comprised in a test line of a LFD. In certain embodiments, a GLL-capture molecule

comprises the same sequence as an aptamer comprised in an insulin probe. In methods relating to a lateral flow assay device of the third aspect, the method comprises isolation of a test line comprised in a detection region of a lateral flow assay device after applying sample and after incubation. Methods of isolation of a test line comprised in a detection area will be known to the skilled person and include, but are not limited to, cutting a detection region of a lateral flow assay device to isolate a test line. In methods of detecting insulin relating to a lateral flow assay device of the third aspect, the methods further comprise releasing glucose from a GLL captured on a test line isolated from a detection region of a lateral flow assay device. Methods of releasing glucose from a liposome encapsulating glucose (GLL) are known in the art and include, but are not limited to treating a GLL with a surfactant. A suitable surfactant includes, but is not limited to non-ionic surfactants such as polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)-phenyl ether (also known as Triton X-100), polysorbate 80 (also known as tween-80), or nonylphenoxypolyethoxylethanol (also known as NP-40). In methods of detecting insulin relating to a lateral flow assay device of the third aspect, the methods further comprise assessing the concentration of the glucose released from a GLL. In certain embodiments, assessing comprises a quantitative measurement of a concentration of a test line, eg measuring a concentration of glucose. In certain embodiments assessing may comprise semi-quantitative or qualitative assessment of a test line, eg detection of insulin above a pre-determined threshold. Methods of assessing glucose concentration are known in the art and include, but are not limited to use of a commercially available glucose test strip such and a portable glucose monitor.

[00122] In one embodiment related to a lateral flow assay device of the third aspect, provided is a lateral flow assay device (LFD) for detecting insulin in a biological sample comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
- (iii) a detection region comprising a test line comprising comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein the GLL-capture molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3';

and;

- (iv) an absorbent pad.

[00123] In embodiments related to a lateral flow assay device of the third aspect, provided is a method of detecting insulin in a sample comprising a lateral flow assay device (LFD) for detecting insulin in a biological sample comprising:

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
- (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein the GLL-capture molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3';

and;

- (iv) an absorbent pad;

wherein the method comprises the steps of

- (a) applying a sample to the sample region of the LFD;
- (b) incubating the LFD;
- (c) isolating the test line comprised in the detection region of the LFD;
- (d) releasing the glucose from the GLL captured on the test line; and
- (e) assessing the glucose released from the GLL.

[00124] Method of preparing a liposome encapsulating glucose (eg, preparing a glucose loaded liposome) are not particularly limited and are known in the art. Methods of introducing a molecule (eg an oligonucleotide, antibody or protein) to the surface of a liposome are not particularly limited and are known in the art (Y. Zhao et al., *Biosensors and Bioelectronics*, 2015, 72, 348-354).

[00125] In embodiments related to methods, kits, and lateral flow assay devices of the third aspect, the lateral flow assay device (LFD) is incubated after applying a sample to a sample region for about 2 minutes to about 20 minutes, about 2 minutes to about 15 minutes, or about 2 minutes to about 10 minutes. In certain embodiments, the LFD is incubated for about 10 minutes to about 15 minutes.

[00126] In embodiments related to methods, kits, and lateral flow assay devices of the third aspect, the method has an insulin detection limit of about 0.03 ng/mL.

[00127] In embodiments related to methods, kits, and lateral flow assay devices of the third aspect, the method comprises detecting at least about 85% of insulin in a sample. In certain

embodiments, the method comprises detecting at least about 90% of insulin in a sample. In certain embodiments, the method comprises detecting at least about 95% of insulin in a sample.

[00128] In embodiments related to a lateral flow assay device of the third aspect, a GLL-capture molecule specific for the molecule comprised on the surface of a GLL may be immobilized to the test line comprised in the detection region by any suitable conditions, including biotin-streptavidin affinity reaction. In certain embodiments, biotin is conjugated to a GLL-capture molecule and streptavidin is used to immobilize the GLL-capture molecule to a test line.

[00129] In certain embodiments, an insulin probe further comprises magnetic particles. Magnetic particles conjugated to an insulin probe for use according to the third aspect may be used for purification in preparing an insulin probe bound to a liposome.

[00130] Embodiments related to methods, kits, and lateral flow assay devices of the third aspect, may further comprise a control component comprised in a sample pad or a conjugate pad and a control component binding molecule immobilized to a control line in a detection region of a lateral flow assay device. In related embodiments, a detection region of a lateral flow assay device (LFD) is configured such that a sample flows past a test line before a control line. Related embodiments may further comprise inspection of the signal of a control line to confirm valid operation of a lateral flow assay device. Inspection may comprise visual confirmation of signal on a control line.

[00131] Kits relating to a lateral flow assay device of the third aspect are disclosed. Kits may comprise a lateral flow assay device wherein an insulin probe is provided, wherein the insulin probe and glucose loaded liposome (GLL) are provided prepared for applying to a sample pad or a conjugate pad of a lateral flow assay device (LFD). In certain embodiments, an insulin probe and a GLL are provided separately and prepared prior to applying to a sample pad or a conjugate pad of a LFD. In certain embodiments, a non-ionic surfactant is provided in a kit relating to a LFD of the third aspect.

[00132] The methods, kits, and lateral flow assay devices are suitable for measuring insulin obtained from a subject at any time. In certain embodiments relating to any lateral flow assay device herein disclosed, a sample is obtained from a subject, wherein the subject has abstained from food and/or beverage for at least about 8 to about 12 hours. In certain embodiments relating to any lateral flow assay device herein disclosed, a sample is obtained from a subject, wherein the subject has abstained from food and/or beverage for at least about 6 to about 8 hours. In certain embodiments, a sample is obtained from a subject, wherein the subject has abstained from food and/or beverage for at least about 2 to about 5 hours. In certain embodiments, a sample is obtained from a subject, wherein the subject has abstained from food and/or beverage for less than about 60 minutes to less than about 1 minute. Abstaining from food and/or

beverage may comprise standard fasting conditions known to the skilled person, eg abstaining from food and beverage (other than water) for a period of time. The skilled person will be familiar with fasting blood tests and the standards applied thereto, which are suitable for preparation of a subject prior to obtaining a sample. For example, fasting conditions may comprise abstaining from food and beverage (other than water) for a number of hours, eg about 8 to about 12 hours. In certain embodiments, a sample may be obtained from a non-fasting subject.

[00133] In a further embodiment, the disclosure provides a method wherein insulin is detected in the morning. In other embodiments, insulin is detected before a meal in the morning. In another embodiment, insulin is detected after a meal in the morning. In certain embodiments, insulin is detected before midday. In another embodiment, insulin is detected at midday. In another embodiment, insulin is detected after a meal at midday. In a further embodiment, insulin is detected before a meal at midday. In certain embodiments, insulin is detected in the evening. In another embodiment, insulin is detected after a meal in the evening. In another embodiment, insulin is detected before a meal in the evening.

[00134] In methods relating to a lateral flow assay device related to the first aspect or second aspect, a test line is assessed. In certain embodiments, assessing comprises a quantitative measurement of the molecules captured on a test line. In certain embodiments assessing may comprise semi-quantitative or qualitative assessment of a test line, eg detection of insulin above a pre-determined threshold. Suitable means of assessing a test line will depend on the signal generated by a test line. For example, assessing may comprise quantitatively measuring the signal from, for example, a fluorescent dye or a colloidal metal. Assessing may be carried out by a smartphone. In certain embodiments, assessing may comprise use of a portable fluorescence meter. Commercially available devices for measuring a signal from a lateral flow assay device will be familiar to the skilled person. In the methods relating to a lateral flow assay device related to the third aspect a test line may be assessed by measuring glucose with a suitable device, for example, a glucose meter.

[00135] In certain embodiments, the insulin level is assessed and/or tracked using a smartphone.

[00136] In certain embodiments related to the second aspect or third aspect, in the methods, kits, and lateral flow assay devices herein described, the detection of insulin may be described as “proportional” to the signal assessed from a test line of a lateral flow device. By proportional it is meant that the signal provided by the assay becomes larger or smaller when the amount of insulin in the sample becomes larger or smaller, respectively. In certain embodiments related to the first aspect, the methods, kits, and lateral flow assay devices herein described, the detection of insulin may be described as “inversely proportional” to the signal assessed from a test line of a lateral flow device. By inversely proportional it is meant that the signal provided by the assay

becomes larger or smaller when the as the amount of insulin in the sample becomes smaller or larger, respectively.

[00137] A lateral flow assay device may be comprised in a suitable housing. The housing may be configured to enclose the device, wherein the housing includes a port allowing access to a sample region and a window allowing visual and/or instrumental access to a test line and control line, if present, comprised in a detection region. The housing may include marking including for example "T" indicating a test line comprised in a detection region and, if applicable, "C" indicating a control line comprised in detection region.

[00138] **Table 1: Exemplary Sequences for Insulin Detection**

|                                                                                  |                                                                                                                                      |
|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Insulin probe comprising an insulin aptamer and a control component              | 5'-GGT GGT GGG GGG GGT TGG TAG GGT<br>GTC TTC TTT TTT TTT TTT TTT TT-3'                                                              |
| Nucleotide sequence specific for an insulin aptamer                              | 5'-GAA GAC ACC CTA CCA ACC CCC CCC<br>ACC ACC-3'.                                                                                    |
| Insulin probe comprising an insulin aptamer and a RCA capture sequence           | 5'-GGT GGT GGG GGG GGT TGG TAG GGT<br>GTC TTC CTCAC TTCAA TTCAT CTGAC-3'                                                             |
| Circular RCA template for insulin                                                | 5'-TTGAA GTGAG AAAAC CCAAC CCGCC<br>CTACC CAAAA GTCAG ATGAA-3'<br><br>5'-TTGAA GTGAG AAAAC CCAAC CCGCC<br>GTTGG GTTTT GTCAG ATGAA-3' |
| Insulin aptamer                                                                  | 5'-GGT GGT GGG GGG GGT TGG TAG GGT<br>GTC TTC-3'                                                                                     |
| Molecule Comprised on surface of a liposome (eg comprised on the surface of GLL) | 5'-GAA GAC ACC CTA C-3'                                                                                                              |

[00139] All references cited herein, including patents, patent applications, publications, and databases, are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not.

[00140] Further preferred embodiments of the disclosure will now be described, by way of example only, with reference to the accompanying drawings.

## EXAMPLES

### [00141] Competitive assay-based LFD

[00142] An exemplary LFD relating to the first aspect comprises a sample region, a conjugation pad, a nitrocellulose (NC) membrane and an adsorbent pad assembled on a backing

plate (Fig. 1). To detect insulin, the insulin probe is applied to the conjugation pad. The sample is applied to the sample region where it flows through the LFD via capillary action. In the presence of insulin, the insulin binds to the insulin probe and rest of the unreacted sample flows to the adsorbent pad, which acts as sink at the end of the LFD. When insulin is present in the sample, it competes with the aptamer binding molecule immobilized on the test line (T-line) for binding to the insulin probe, wherein the insulin probe has higher affinity for insulin than the aptamer binding molecule has for the insulin probe. Accordingly, as the concentration of insulin increases, the intensity of the signal on the T-line decreases. With or without insulin in a sample, the insulin probe is captured by the control component binding molecule immobilized on the control line (C-line). Accordingly, signal on the C-line confirms valid operation of the lateral flow assay. The intensity of the T-line is inversely proportional to the concentration of insulin in the sample. The signal corresponding to the concentration of insulin may be assessed by suitable means, including as a colour readout or a fluorescent readout. The intensity of the T-line may be assessed using a strip reader (e.g. Ax-2x Lateral Flow Reader), portable fluorescence meter or a smartphone-based application.

[00143] **Example 1: Experimental Procedures for a Competitive assay-based LFD**

[00144] **Materials for Competitive assay-based LFD of Example 1**

[00145] Gold nanoparticles (AuNPs), phosphate buffer saline (PBS) (0.01 M, pH 7.4), streptavidin, insulin, uric acid, ascorbic acid, sucrose, polyethylene glycol (PEG), Tween-20, bovine serum albumin (BSA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and serum were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Sample pad, absorbent pad, conjugation pad, nitrocellulose (NC) membrane and backing card were acquired from Shanghai Kinbio Tech. Co., Ltd., China. The sequences of oligonucleotides used in Example 1 experiments were as follows (also in Table 1):

[00146] **Insulin probe of Example 1:** Gold nanoparticles (AuNPs) modified with insulin aptamer (AuNPs-5'SH-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3'), or insulin aptamer modified with fluorescence dye Texas red (5'Texas red-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3').

[00147] **T-line of Example 1:** aptamer binding molecule specific for aptamer comprised in insulin probe (5' Biotin-GAA GAC ACC CTA CCA ACC CCC CCC ACC) (referred to as "DNA 1").

[00148] **C-line of Example 1:** control component binding molecule (5' Biotin AAA AAA AAA AAA AAA AAA AA-3') (referred to as "DNA 2").

[00149] Instruments for insulin preparation such as the Strip Guillotine Cutter ZQ2002, Dispenser HM3035 and drying oven PH050A were purchased from Shanghai Kinbio Tech. Co.,

Ltd., China. All the aptamer sequences were ordered from Sangon Biotech., China and lyophilized powder was dissolved in PBS buffer (0.01 M, pH 7.4).

**[00150] Preparation of streptavidin conjugated DNA 1 and DNA 2 of Example 1**

[00151] DNA 1 and DNA 2 were immobilized on NC membrane using streptavidin-biotin affinity reaction. 35  $\mu$ L of 1 mg/mL streptavidin solution were added to 35  $\mu$ L of DNA 1 and incubated at 4°C for 2 hours. Thereafter, 30  $\mu$ L PBS (0.01 M, pH 7.4) was added and this solution was used to make the test line (T-line) on NC membrane. Similarly, the control line (C-line) was made by adding DNA 2 instead of DNA 1. The T-line and C-line were dispersed on the NC membrane with interval of 5 mm using dispenser HM3035. These lines were marked as test line (T-line) and control line (C-line) respectively. The NC membrane was incubated at 37°C for 1 hour and stored in desiccator until further use.

**[00152] Preparation of insulin probe of Example 1**

[00153] Insulin probe was activated by adding 3  $\mu$ L of TCEP (5 mg/mL) into 3  $\mu$ L of 100  $\mu$ M probe and incubated for 1 hour at 4°C. 5 mL of gold nanoparticles (AuNPs) (pH 8.5) were added to 80nM aptamer solution and react overnight at room temperature to obtain the activated insulin probe. Thereafter, NaCl (0.1 M) solution was added to the mixture under continuous shaking by adding 10  $\mu$ L NaCl every 20 minutes until the final concentration of NaCl reached 80 mM. Excess aptamer was removed by centrifugation at 10,000 rpm for 15 minutes and resuspend in 0.01 M PBS (pH 7.4) containing 0.5% PEG, 5% sucrose, 0.25% Tween 20, and 1% BSA. The insulin probe (AuNPs–Apt) were stored in the dark at 4 °C for future use.

**[00154] Preparation of a Competitive assay-based LFD of Example 1**

[00155] The sample pad was treated using sample treatment buffer (0.01 M PBS buffer (pH 7.4) containing 1 % BSA, 0.25 % Tween 20, and 2 % sucrose) and incubated at 37°C for 2 hours. The insulin probe conjugated AuNPs were used to treat conjugation pad and it was incubated at 37°C for 1 hour. The sample pad and conjugation pad were stored in the desiccator until further use. The NC membrane, sample pad, conjugation pad and absorbent pad were pasted on backing card with 2 mm overlapping each other. Then 4.0 mm wide strips were cut using the Strip Guillotine Cutter ZQ2002.

[00156] For a colorimetric signal readout, the AuNP labelled insulin aptamer is used as the insulin probe.

[00157] For a fluorescence signal readout, the Texas red labelled insulin aptamer is used as the insulin probe.

[00158] **Optimization of parameters for a Competitive assay-based LFD of Example 1**

[00159] The parameters for a Competitive assay based LFD were optimised. The parameters included the temperature used to treat the backing plate, ratio of DNA 1 on the T-line to streptavidin (2:1, 1:1, 1:3, 1:6, 1:9); ratio of DNA 2 on the C-line to streptavidin (2:1, 1:1, 1:3, 1:4, 1:6), and the response time (2 to 10 minutes). The intensity of test strips was recorded for detection of 50 ng/mL of insulin. **Figure 2** illustrates the optimization result of the streptavidin proportion to DNA 1 and DNA 2.

[00160] The optimal temperature for incubating the backing plate was observed at 37°C for 1 hour while no test line was observed when the incubation temperature was 60°C. The high temperature of 60°C may have decomposed the insulin aptamer. The ratio of DNA 1/DNA 2 at 1:3, DNA 1/ streptavidin at 1:3 and DNA 2/streptavidin at 1:1 provided the highest colorimetric intensity (Fig. 2A and Fig. 2B). It was observed that the intensity ( $I_{c-line}/I_{t-line}$ ) increased when the incubation time increase from 2 to 10 minutes, and the maximum intensity was observed when the incubation time was 6 minutes. There was no significant difference in intensity between 6 minutes and 10 minutes. Thus, 6 minutes was considered an optimized incubation time.

[00161] **Optimization of gold nanoparticles (AuNPs) under different conditions for Example 1**

[00162] The insulin probe was conjugated to AuNPs using conjugation buffer containing 0.01 M PBS (pH 7.4), 2 % sucrose, 1 % BSA, 0.25 % Tween 20 and 0.5 % PEG. This conjugation buffer facilitates the release of AuNPs-aptamer from the conjugation pad and reduces the non-specific binding of AuNPs-aptamer to the NC membrane. The components of the buffer move along the membrane and block it naturally without requiring an additional blocking step. Using the zeta-sizer the size distribution of AuNPs was measured and it was found that the size of AuNPs was 65 nm (**Fig. 3**) and the zeta potential was  $-20.76 \pm 0.48$  mV.

The concentration of AuNPs was calculated using the following formula below:

$$\text{Molarity (i)} = \frac{\text{conc. of AuNPs in mg mL}^{-1}}{\frac{4}{3} \pi R^3 \rho N_A} \text{ AuNPs}$$

Where, R = radius of AuNPs,  $\rho$  = density of AuNPs and  $N_A$  = Avogadro Number

It was found that the concentration of AuNPs was 0.082  $\mu\text{M}$ .

[00163] The loading density of the aptamer on AuNPs was calculated by measuring the absorbance before and after using UV-vis spectroscopy (Jin et al., *Biosensors and Bioelectronics*, 2017, 90, 525-533). The absorbance difference was then converted to concentration of DNA. The

loading density was calculated by calculating the ratio of concentration of DNA to concentration of AuNPs.

$$\text{Loading density of aptamer} = \frac{\text{Molarity of DNA}}{\text{Molarity of AuNPs}} \quad (\text{ii})$$

Concentration of AuNPs from equation (i) was 0.082  $\mu\text{M}$ . According to Beer-Lambert law:

$$A = \epsilon_m c l \quad (\text{iii})$$

where,  $\epsilon_m$  = molar extinction coefficient,  $c$  = concentration of sample and,  $l$  = path length

$\epsilon_m$  for aptamers is 0.027, therefore, concentration of insulin probe was 4.07  $\mu\text{M}$ .

[00164] Using equation (ii), it was found that the loading density was  $49.6 \pm 4.5$  or  $\sim 50$  aptamer per particle.

[00165] Insulin aptamer sequence was conjugated to AuNPs and the effect of different pH (3, 6, 7, 9, 11 and 12) and concentrations of AuNPs (0, 0.008, 0.032, 0.056, 0.08, 0.16, 0.32  $\mu\text{M}$ ) was studied. 3 ng/mL insulin was added to the sample pad and incubated for 10 minutes. The optimum pH for binding of aptamer to AuNPs was 7.0 (Fig. 4A) and the optimum concentration of AuNPs was 0.08  $\mu\text{M}$  (Fig. 4B).

[00166] When AuNPs and aptamer exist simultaneously in a solution, NaCl can help to form a stable Au-S bond between the thiol group tagged to the aptamer and the AuNPs to increase the load capacity. To investigate the influence of NaCl, different concentrations of NaCl were added to the solution of AuNPs and aptamers. With the increase of NaCl concentration from 0 to 100  $\mu\text{M}$ , the intensity decreased, signifying that more aptamers had attached to the surface of the AuNPs. When the concentration of NaCl reached 80  $\mu\text{M}$ , the load capacity of aptamer was almost saturated. Thus, 80  $\mu\text{M}$  of NaCl is the optimum concentration and was used for detection (Fig. 5).

#### [00167] **Calibration curve for detection of insulin by colorimetric assay for Example 1**

[00168] About 50  $\mu\text{L}$  of insulin sample was dropped onto the sample pad to react with insulin probe in the conjugation pad. After 10 minutes at room temperature, the intensity of the T-line was quantified by the ImageJ software. To obtain a calibration curve, PBS containing different concentrations of insulin 0, 0.01, 0.1, 0.2, 0.5, 1, 3, 5, 10, 20, 50, 100, and 200 ng/mL was prepared and used as the insulin sample (See Figure 6). The intensity of the T-line was calculated using ImageJ software and calibration curve was plotted for insulin concentrations. The performance of the competitive assay-based LFD was evaluated against the conventional ELISA kit.

[00169] It was observed that the optical signal increased linearly with the concentration of insulin. Under the optimized conditions as described above, the calibration curve for detection of insulin in PBS was obtained (Fig. 6A). As shown in Fig. 6, a competitive assay-based LFD can be used for detection of insulin with the linear range of 0.01 ng/mL to 150 ng/mL and the detection limit was 0.01 ng/mL, which is within the detection limit of insulin in medical research (0.35 ng/mL). The initial results of the colorimetric competitive assay-based LFD for the detection of insulin in buffer solution is illustrated in Fig. 6B.

[00170] **Performance evaluation of a Competitive assay-based LFD of Example 1 against ELISA**

[00171] The performance of a competitive assay-based LFD (AuNPs) for the detection of insulin in buffer with the concentration of 1, 2, 5, 10, 20 ng/mL was evaluated using the commercially available human insulin ELISA kit. As shown in Fig. 7, the competitive assay-based LFD has comparable performance to the ELISA with similar trend when the insulin concentration increases. However, the present competitive assay-based LFD has a quick response time (less than 10 minutes) and is better suited for point-of-care testing.

[00172] **Specificity, Selectivity and Stability of a Competitive assay-based LFD of Example 1**

[00173] The specificity and selectivity of a competitive assay-based LFD (AuNPs) was tested using glucose, ascorbic acid and uric acid instead of insulin (Fig. 13). The test strips were stored in desiccated environment and their stability was checked at different time intervals. Figure 13 corresponds to incubation of the LFD for 15 minutes.

[00174] With reference to Figure 13A, color intensity of on the test line ("t-line") was observed after adding insulin (3 ng/mL), glucose (1 mg/mL), uric acid (0.1 mg/mL), human serum albumin (HAS, 50 mg/mL), or IgG (20 mg/mL) to a competitive assay-based LFD. With reference to Figure 13A, color intensity intensity on the t-line after adding glucose (1 mg/mL), uric acid (0.1 mg/mL), human serum albumin (HAS, 50 mg/mL), or IgG (20 mg/mL), in the presence of insulin (3 ng/mL), to a competitive assay-based LFD. The normal values of uric acid in blood for women are 2.5 to 7.5 mg/dL and for men 4.0 to 8.5 mg/dL. A normal fasting (no food for eight hours) blood sugar level is between 70 to 99 mg/dL. The normal concentration of HSA in blood serum is 35 to 50 mg/mL. The normal concentration of IgG in adult blood serum 7 to 16 mg/mL. It was observed (13 A) that the color intensity was weaker for insulin than that for other molecules present in human blood, which may interfere with the detection of insulin from blood sample, blood suggesting that the LFD herein described is specific to insulin. The specificity for insulin was confirmed by detecting insulin in the presence of an interference molecule with an LFD herein

described, where a color intensity decrease was observed (13 B), which confirms that an LFD herein described was selective for insulin.

**[00175] Evaluation of a Competitive assay-based LFD of Example 1 using saliva and blood samples**

[00176] A competitive assay-based LFD (AuNPs) was prepared according to the above optimized conditions. It has been reported that the concentration of insulin in saliva is about 10% of that measured in serum (B. Fabre et al., Endocrine Connections, 2012, 1, 58-61). Saliva was spiked with 0.03 ng/mL, 0.3 ng/mL and 1 ng/mL of insulin. 50  $\mu$ L of saliva was added to the sample pad of each LFD. The T-line and C-line were visible in 6 minutes (Fig. 8). A lower colour intensity on the T-line was observed when insulin in saliva was higher. The recovery of insulin from the saliva sample was about 81 to 97% with CV%  $\pm$ 4.6%. The recovery of insulin was calculated by comparing the spiked concentration of insulin and the amount of insulin detected. For example, if the spiked insulin concentration was 1 ng/mL and the detected insulin concentration was 0.9 ng/mL then the recovery would be 90%.

[00177] A competitive assay-based LFD was prepared according to the above optimized conditions for testing blood samples. The blood samples were diluted 100 times and spiked with 0.01 ng/mL, 0.02 ng/mL, 0.04 ng/mL, 0.2 ng/mL and 1 ng/mL of insulin (Fig. 9). The recovery of insulin from the blood sample was about 86 to 101% with CV%  $\pm$ 7.3%. To rule out the effect of red blood cells in whole blood samples, a membrane with a size of 0.44  $\mu$ m was specifically added onto the sample pads before blood samples were loaded.

**[00178] Calibration curve for detection of insulin by fluorescence in a Competitive based-assay LFD (Texas Red) of Example 1**

[00179] The optical signal increased linearly with the concentration of insulin (Fig. 10). The calibration curve for the detection of insulin in PBS was obtained under the above optimized conditions (Fig. 10B). As shown in Fig. 10, a competitive assay-based LFD can be used for the detection of insulin with a linear range of 0.01 ng/mL to 100 ng/mL and the detection limit was 0.01 ng/mL, which is within the detection limit of insulin in medical research (0.35 ng/mL). We also evaluated the performance of the competitive assay-based LFD using different concentrations of insulin (0.5, 1, 2, 4, 8 ng/mL) and comparing the results to the commercially available human insulin ELISA kit. As shown in Fig. 10C the performance of the competitive assay-based LFD (Texas Red) was comparable to ELISA. However, the competitive assay-based LFD has a rapid response time of less than 10 minutes and is better suited for point-of-care testing.

**[00180] Example 2: Experimental Procedures for a Competitive assay-based LFD**

**[00181] Materials for Competitive assay-based LFD of Example 2**

[00182] Gold nanoparticles, phosphate buffer saline (PBS) (0.01 M, pH 7.4), streptavidin, insulin, uric acid, ascorbic acid, sucrose, polyethylene glycol (PEG), Tween-20, bovine serum albumin (BSA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and serum were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Sample pad, absorbent pad, conjugation pad, nitrocellulose membrane and backing card were acquired from Shanghai Kinbio Tech. Co., Ltd., China. All the aptamer sequences were ordered from Sangon Biotech., China in lyophilized powder and was dissolved in PBS buffer (0.01 M, pH 7.4). The sequences of oligonucleotides used in Example 2 experiments were as follows (also in Table 1):

[00183] **Insulin probe of Example 2:** 5'-SH (C<sub>6</sub>)-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TT-3'.

[00184] **T-line of Example 2:** 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-biotin-3'.

[00185] **C-line of Example 2:** 5'-AAA AAA AAA AAA AAA AAA AA-biotin-3'.

[00186] Instruments for insulin preparation like Strip Guillotine Cutter ZQ2002, Dispenser HM3035 and drying oven PH050A were purchased from Shanghai Kinbio Tech. Co., Ltd., China. The nanoparticles were characterized using UV-visible spectroscopy and zeta sizer. The quantitative determination of insulin was carried out using Ax-2x lateral flow reader (Axxin, Victoria, Australia).

**[00187] Preparation of streptavidin conjugated DNA 1 and DNA 2 of Example 2**

[00188] DNA 1 and DNA 2 were immobilized on nitrocellulose (NC) membrane using streptavidin-biotin affinity reaction. Streptavidin (1 mg/mL) solution was added to DNA 1 (100  $\mu$ M) and incubated at 4°C for 2 hours. This solution was used to make T-line on NC membrane. Similarly, C-line was made by adding DNA 2 (100  $\mu$ M) instead of DNA 1. T-line and C-line were dispersed on the NC membrane with interval of 5 mm using dispenser HM3035. The NC membrane was incubated at 37°C for 2 hours. The NC membrane was and in desiccator until further use.

**[00189] Preparation of insulin probe of Example 2**

[00190] Insulin probe was activated by adding 6  $\mu$ L of TCEP (10 mg/mL) into 3  $\mu$ L of 100  $\mu$ M probe and incubated for 2 hours at 4°C. The activated probe was added to 1 mL of AuNPs (pH 7.0). Then the solution was incubated at room temperature overnight. After that NaCl (1 M) solution was added to the mixture under continuous shaking by adding 10  $\mu$ L NaCl every 20

minutes till the final concentration of NaCl reached 80 mM. The mixture was aged for 12 hours at room temperature. Excess aptamer was removed by centrifugation at 10 000 rpm for 15 minutes and resuspend in 0.01 M PBS (pH 7.4) containing 5% sucrose, 0.1% Tween 20, and 1% BSA. AuNPs–Apt were stored in dark at 4°C for future use.

**[00191] Preparation of a Competitive assay-based LFD of Example 2**

[00192] The sample pad was treated using sample treatment buffer (0.01 M PBS buffer (pH 7.4) containing 0.5 % PEG, 5 % sucrose, 1 % BSA and 0.25 % Tween-20 and incubated at 37°C for 2 hours. Conjugation pad treatment buffer was pre-treated using 0.01 M PBS buffer (pH 7.4) containing 10 % sucrose, 0.25 % Tween-20, and 0.01 % NaN<sub>3</sub> and dried at 37°C for 2 hours. Then insulin probe conjugated AuNPs were used to treat conjugation pad and incubated at 37°C for 2 hours. In case of fluorescent based LFDs, no conjugation pad was used. The sample pad and conjugation pad were stored in desiccator until further use. Nitrocellulose membrane (NC membrane), sample pad, conjugation pad and absorbent pad were pasted on backing card with 2 mm overlapping each other. Then 5.0 mm wide strips were cut using Strip Guillotine Cutter ZQ2002.

**[00193] Optimization of parameters for a Competitive assay-based LFD of Example 2**

[00194] Different concentrations of DNA 1 and DNA 2 (0, 25, 50, 75, 100 µM) were used for C-line and T-line. For fluorescent based LFDs DNA 1 and DNA 2 (0, 2, 4, 6, 8 and 10 µM) were used to optimize C-line and T-line concentration. Different concentrations of insulin probe (1, 10, 20, 30 40 and 50 µM) were conjugated to AuNPs (0.08 µM, pH 7.0). The concentration and pH of AuNPs was optimized. NaCl prevents agglomeration of AuNPs, therefore its concentration is quite important. 0-100 mM NaCl concentration was added to prevent aggregation of AuNPs. Streptavidin helps in binding of DNA 1 and DNA 2 to NC membrane through biotin-streptavidin affinity reaction. Streptavidin was mixed with DNA 1 and DNA 2 in different proportions (0:1, 1:1, 1.5:1, 2:1, 2.5:1). For fluorescent based LFDs different concentrations of insulin probe (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µM) were mixed with 50 µL PBS (0.01 M, pH 7.4) and incubated at room temperature for 30 minutes. Incubation time also play important role in insulin concentration detection. Therefore, insulin probe (8 µM) was mixed with 70 µL insulin (0.3 ng/mL) and incubated at room temperature for different time intervals (5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes). The mixture was applied to sample pad and strips were incubated for 12 minutes. The response time was studied from 1 minute to 15 minutes.

**[00195] Specificity, Selectivity, Stability and Repeatability of a Competitive assay-based LFD of Example 2**

[00196] The specificity of the LFD was detected using IgG, uric acid, glucose, BSA and HSA (300 ng/mL) instead of insulin (0.3 ng/mL). The selectivity of the LFD was determined by mixing IgG, uric acid, glucose, BSA, HSA and insulin and dropping the analytes on sample pad. For fluorescent based LFDs, specificity and selectivity was studied using IgG, uric acid, glucose, BSA and HSA (300 ng/mL) as interfering agents. The test strips were stored in desiccated environment and their stability was checked at different time intervals (0-30 days). The repeatability of the assay was determined by measuring the relative intensity (T/C) of LFD test strips by adding insulin (0.5 ng/mL) to the strips. The experiments were repeated 10 times for measuring the repeatability.

[00197] **Using Smartphone as signal readout module**

[00198] Samsung Galaxy S10 was used as the main part. The smartphone has a high resolution (3040 × 1440 p) and the screen pixel density is 550 ppi. The smartphone has three rear cameras: 12 MP telephoto camera, 12 MP wide-angle camera and 16 MP ultra-wide camera. Therefore, the choice of this smartphone as image processing, data acquisition and signal readout was appropriate. The black smartphone accessory (102 × 90 × 72 mm) (Fig. 29) was designed by SolidWorks software and fabricated using a 3D printer (Ultimaker 3). The attachment module included a sample slot and the flash of smartphone was selected as the source of light. The design of the accessory box helped to provide a homogeneous optic field and better focusing distance, such that a high-quality image could be captured. The easy to operate and user-friendly software was developed using Java programming language which provided the functions of image processing, data analysis and cloud data storage. After the LFD was inserted in the accessory box, the smartphone camera was used to take the picture and the results were immediately displayed on the screen. All the data was stored in the cloud named, Insulin platform, where it can be analysed and referred to later. Once registered to the cloud, one can then have access to the software where data management can be carried out. This feature could be very useful for clinical monitoring as big data gets stored in the cloud.

[00199] We designed a software system which consists of two parts: smartphone and cloud server. The user can use a smartphone to take a picture, then the App will automatically calculate the intensity of Insulin and upload the result to a cloud server.

[00200] **Introduction of the Smartphone App**

[00201] **Platform:** Samsung S10

[00202] **Development Environment:** Windows, Android Studio

[00203] **Programming Language:** Java

[00204] **Implementation:** Currently, the smartphone App works with a 3D-printed black box (Fig. 29). The box is used to provide a stable illumination environment to improve precision. The smartphone-based App is used to record the images, evaluate the results and upload the results to the server. The steps are as follows. 1) Insert the LFD test strip into the notch inside the black box. The function of the black box is to eliminate the effect of light from the environment. 2) Insert the smartphone into the notch located on top of the box. 3) Launch the App and adjust the smartphone slightly to ensure C/T lines are located inside the white rectangles. 4) Click the “take picture” button to take a photo of the LFD test strip. Once the image is captured the App will be used to run a custom-developed digital image processing algorithm to calculate the concentration of the test strip. The steps of the automated image processing are as follows:

[00205] 1) The image will be first converted to grayscale and then it will be processed to extract the locations and relative intensities of the lines on the test strip.

[00206] 2) The area of C line and T line will be clipped from the original image to calculate the grayscale intensity of C line and T line.

[00207] 3) For each area, the maximum value of the average column pixel intensity per row vector will be taken and every pixel that is less than 90% of this value will be zeroed to remove the parts of the image which do not carry any useful information, such as the background. This will leave us with only non-zero values for pixels that are part of the test strip itself. Then the grayscale intensity of C line and T line are obtained by calculating the average of the rest grayscale pixels.

[00208] 4) Based on the relative intensity of C line and T line, the concentration is calculated based on a linear model. The linear model is obtained from the LFD test strips with accurate concentrations and it has the following format:  $\text{concentration} = A \times \text{relative intensity} + B$ , where A and B are learnt from the accurate LFD test strips. According to this linear model, once we know the relative intensity, we can know the concentration of the test strip.

[00209] 5) The test is invalid if C-line or T-line is absent, or the concentration is out of range. In this case, the App will show “Invalid, take another photo”. If the result is valid, the App will show the result and upload the result as well as the image to the cloud server.

[00210] Using wireless communication (e.g., Wi-Fi, GSM, CDMA, etc.), the App can upload the result and image to the cloud server. If uploaded successfully, the App will pop up a message showing “upload successfully”. In case wireless connectivity is lost, all the images would be stored in the internal memory of the smartphone.

[00211] **Application of LFD for detection of insulin levels in clinical samples from Example 2**

[00212] The insulin concentrations were checked in saliva samples. The samples (70  $\mu$ L) were added to sample pad. The strips were incubated for 12 minutes and quantified using Ax-2x lateral flow reader (Axxin, Victoria, Australia).

[00213] **Results and Discussions of Example 2**

[00214] **Optimization of the probe concentration for colorimetric LFDs of Example 2**

[00215] The probe concentration was varied from 1, 10, 20, 30, 40 and 50  $\mu$ M. The intensity saturated after 30  $\mu$ M as most of the probe had bonded to T and C-lines (Fig. 15). Therefore, 30  $\mu$ M was taken as the optimum concentration for insulin binding aptamer.

[00216] **Optimization of the probe concentration for fluorescent based LFDs of Example 2**

[00217] The insulin probe labelled with Texas red on its 5' end was used as the probe. The C and T-line were prepared by adding 75  $\mu$ M poly (A) tail and c-DNA for insulin probe respectively to the NC membrane. Different concentrations of insulin probe (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10  $\mu$ M) were mixed with 50  $\mu$ L PBS (0.01 M, pH 7.4) and incubated at room temperature for 30 minutes. The mixture was applied to sample pad and strips were incubated for 12 minutes. The readings were taken using Axxin reader. It is clear from Fig. 16 that 8  $\mu$ M insulin probe concentration can be taken as the optimum concentration as after this the relative intensity becomes saturated. This is because there are no more free aptamers to bind to the C and T-line.

[00218] **Optimization of DNA 1 and DNA 2 concentration for colorimetric LFDs of Example 2**

[00219] The complementary sequence or cDNA has greater affinity for binding to the AuNPs-aptamer (Jauset-Rubio et al. 2016). DNA 2 at the C-line was kept constant at 100  $\mu$ M and streptavidin (1 mg/mL) was used in 1:1 to DNA 1 and DNA 2. Insulin (3 ng/mL) was added to the sample pad and strips were incubated for 12 minutes. Different concentrations of DNA 1 (0, 25, 50, 75, 100  $\mu$ M) were added at the T-line. It was observed that 75  $\mu$ M could be taken as optimum concentration as above this concentration the intensity of T-line is reduced (Fig. 17). This is because the insulin aptamer binds to c-DNA at T-line and no more free aptamer was available to bind which reduces the intensity of T-line.

[00220] The ratio of DNA 1 and DNA 2 was kept at 1:1 to streptavidin (1 mg/mL). Using 75  $\mu$ M as T-line concentration, different concentrations of DNA 2 (0, 25, 50, 75, 100, 125  $\mu$ M) were used to form the C-line. Insulin (3 ng/mL) was added to the sample pad and strips were incubated for 12 minutes. It was observed that 75  $\mu$ M could be taken as optimum concentration as after this

concentration, the intensity of the C-line was reduced (Fig. 17). This may be because after this concentration, all the base pairs in poly (A) tail has been bound to poly (T) tail of insulin aptamer.

**[00221] Optimization of DNA 1 and DNA 2 concentration for fluorescent based LFDs of Example 2**

[00222] DNA 2 at the C-line was kept constant at 100  $\mu\text{M}$  and streptavidin (1 mg/mL) was used in 1:1 to DNA 1 and DNA 2. Insulin (0.3 ng/mL) was added to the sample pad and strips were incubated for 12 minutes. Different concentrations of DNA 1 (0, 2, 4, 6, 8 and 10  $\mu\text{M}$ ) were added at the T-line. It was observed that 6  $\mu\text{M}$  could be taken as optimum concentration as after this concentration, the intensity of T-line was reduced (Fig. 18). This is because the insulin aptamer binds to c-DNA at T-line and no more free aptamer was available to bind which reduces the intensity of T-line.

[00223] The ratio of DNA 1 and DNA 2 was kept at 1:1 to streptavidin (1 mg/mL). Using 6  $\mu\text{M}$  as T-line concentration, different concentrations of DNA 2 (0, 2, 4, 6, 8 and 10  $\mu\text{M}$ ) were used to form the C-line. Insulin (0.3 ng/mL) was added to the sample pad and strips were incubated for 12 minutes. It was observed that 75  $\mu\text{M}$  could be taken as optimum concentration as after this concentration, the intensity of C-line was reduced (Fig. 18). This may be because after this concentration all the base pairs in poly (A) tail has been bound to poly (T) tail of insulin aptamer.

**[00224] Optimization of incubation time for fluorescent based LFDs of Example 2**

[00225] The insulin probe labelled with Texas red on its 5' end was used as the probe. The C-line and T-line were prepared by adding 6  $\mu\text{M}$  poly (A) tail and c-DNA for insulin probe respectively to the NC membrane. The sample pad was pre-treated with sample pad treatment buffer containing 0.01 M PBS (pH 7.4), 0.5 % sucrose, 1 % BSA and 0.25 % Tween-20. No conjugation pad was used during the preparation of fluorescent test strip. The insulin probe (8  $\mu\text{M}$ ) was mixed with 70  $\mu\text{L}$  insulin (3 ng/mL) and incubated at room temperature for different time intervals (5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes). The mixture was applied to sample pad and strips were incubated for 12 minutes. The readings were taken using Axxin reader. It is clear from Fig. 19 that 25 minutes is the optimum incubation time as during this time all the insulin binds to the probe and further increase in time makes no significant change.

**[00226] Calibration curve for insulin to obtain linear range and detection limit of insulin for colorimetric LFDs of Example 2**

[00227] The insulin binding aptamer (5  $\mu\text{L}$ , 30  $\mu\text{M}$ ) was mixed with 10  $\mu\text{L}$  of TCEP (10 mg/mL) and incubated at room temperature for 1 hour. The mixture was added to 1 mL AuNPs (28.8  $\mu\text{M}$ ) and incubated at 4°C for 24 hours. The solution was aged by adding Tris-HCl (10 mM, pH 8.2) containing NaCl (1M) till the final concentration reached 30 mM. After that 1 % SDS was added

with final concentration of 0.01 %. The mixture was incubated at 4°C for another 24 hours. The excess aptamer was removed by centrifugation at 12, 000 rpm for 15 minutes. The pellets were resuspended in 0.01 M PBS (pH 7.4) containing 5 % sucrose, 0.1 % Tween-20 and 1% BSA. The probe was stored at 4 °C till further use. Different insulin concentrations (0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 30 and 50 ng/mL) (70 µL) were added to test strips and incubated for 12 minutes. The detection for each concentration was repeated 10 times. The readings were recorded with the Axxin reader. It was observed (see Fig. 20) that the relative intensity (T/C) decreased with increasing concentration of insulin ( $R^2 = 0.9597$ ). The limit of detection (LOD) was determined to be 0.01 ng/mL and the detection range was 0.01-1 and 1-10 ng/mL.

**[00228] Calibration curve for insulin to obtain linear range and detection limit of insulin for fluorescent based LFDs of Example 2**

[00229] The insulin probe labelled with Texas red on its 5' end was used as the probe. The C-line and T-line were prepared by adding 6 µM poly (A) tail and c-DNA for insulin probe respectively to the NC membrane. The sample pad was pre-treated with sample pad treatment buffer containing 0.01 M PBS (pH 7.4), 0.5 % sucrose, 1 % BSA and 0.25 % Tween-20. No conjugation pad was used during the preparation of fluorescent test strip. The insulin probe (8 µM) was mixed with 70 µL of different insulin concentrations (0, 0.03, 0.1, 0.5, 1, 3, 5, 10, 30 and 100 ng/mL) and incubated at room temperature for 25 minutes. The mixture was applied to sample pad and strips were incubated for 12 minutes. The readings were taken using Axxin reader. It was observed (see Fig. 21) that the relative intensity (T/C) decreased with increasing concentration of insulin ( $R^2 = 0.9961$ ). The limit of detection (LOD) was determined to be 0.01 ng/mL and the detection range was 0.01-1 ng/mL.

**[00230] Specificity, Selectivity, Stability and Repeatability of colorimetric LFDs of Example 2**

[00231] For the determination of specificity, we tested various interfering agents like HSA, BSA, glucose, uric acid and IgG (300 ng/mL) against insulin (0.3 ng/mL). Each of the solution (70 µL) was added to the sample pad of the LFD test strip and incubated for 12 minutes. The readings were taken with the Ax-2x lateral flow reader and it was observed that the LFDs are specific for insulin. All the experiments were repeated three times for each analyte. The intensities of the HSA, BSA, glucose, uric acid and IgG were equivalent to that of PBS whereas the intensity is minimum for insulin, which shows the strips are specific for insulin (Fig. 22A).

[00232] Similarly, the selectivity of LFDs of Example 2 was determined by using HSA, BSA, glucose, ascorbic acid and IgG in presence of insulin. Each of the analyte (70 µL) was added to the optimized test strips. As shown in Fig. 22B, HAS, BSA, glucose, uric acid and IgG displayed

a basic consistent level of the relative intensity (T/C) compared with the blank solution, only insulin gave specific reduction, which indicated that the LFD test strips were selective for insulin (Fig. 22B).

[00233] To confirm the stability of LFD test strips, the as-prepared strips from the same batch were stored in sealed plastic bags and kept at room temperature in desiccated environment. The assay was conducted with insulin (0.5 ng/mL) at 5 days interval. As shown in Fig. 22C, the relative intensity (T/C) of the test strips is nearly the same over the tested days 0-30 days thus demonstrating the stability of the colorimetric LFDs.

#### [00234] **Specificity, Selectivity, Stability and Repeatability of fluorescent based LFDs of Example 2**

[00235] For the determination of specificity, we tested various interfering agents like IgG, uric acid, glucose, BSA and HSA (300 ng/mL) against insulin (0.3 ng/mL). Each of the solution (70  $\mu$ L) was added to 5  $\mu$ L Texas red labelled aptamer and incubated at room temperature for 25 minutes. Then 70  $\mu$ L of the mixture was applied to the sample pad and incubated for 12 minutes. The readings were taken with the Ax-2x lateral flow reader and it was observed that the LFDs were specific for insulin. All the experiments were repeated three times for each analyte. The intensities of the IgG, uric acid, glucose, BSA and HSA were equivalent to that of PBS whereas the intensity is minimum for insulin, which shows the strips were specific for insulin (Fig. 23A).

[00236] Similarly, selectivity of the fluorescent based LFDs were determined by using IgG, uric acid, glucose, BSA and HSA in the presence of insulin. Each of the solution (70  $\mu$ L) was added to 5  $\mu$ L Texas red labelled aptamer and incubated at room temperature for 25 minutes. Then 70  $\mu$ L of the mixture was applied to the sample pad and incubated for 12 minutes. As shown in Fig. 23B, IgG, uric acid, glucose, BSA and HSA displayed a basic consistent level of the relative intensity (T/C) compared with the blank solution, only insulin gave specific reduction, which indicated that the fluorescent based LFD test strips were selective for insulin (Fig. 23B).

[00237] To confirm the stability of fluorescent-based LFD test strips, the as-prepared strips from the same batch were stored in sealed plastic bags and kept at room temperature in desiccated environment. The assay was conducted with insulin (0.5 ng/mL) at 5 days interval. As shown in Fig 23C, the relative intensity (T/C) of the test strips is nearly the same over the tested days 0-30 days thus demonstrating the stability of the fluorescent based LFDs.

#### [00238] **Application of colorimetric LFDs of Example 2 for detection of insulin levels in human saliva samples**

[00239] To evaluate the practicability and accuracy of the colorimetric LFDs, saliva samples (n= 3) (EC number HC190300) were collected (70  $\mu$ L) at different intervals of the day i.e. fasting

(over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm) and insulin levels were determined on the LFD at about 30 minutes after each meal (Fig 24B). The samples were added to the test strips and incubated for 12 minutes. The results were read using the Ax-2x lateral flow reader (Fig. 24). It was observed that at fasting, insulin levels were low which raised following breakfast and lunch (see Fig. 24A as reported in Daly et al. 1998). The insulin levels in saliva samples were determined using the colorimetric LFD and human insulin ELISA kit. It was observed that the correlation coefficient between both detection samples was 85 %.

**[00240] Application of colorimetric LFDs of Example 2 for detection of insulin levels in human blood samples**

[00241] The blood samples were collected at different intervals of the day i.e. fasting (over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm) and insulin levels were determined on the LFD at about 30 minutes after each meal. A drop of blood (collected by finger prick collection method) was added to the LFD followed by the addition of 40  $\mu$ L of running buffer (PBS 0.01 M, pH 7.4 containing 1 % BSA and 0.5 % Tween-20). The LFD was then incubated for 12 minutes. The results were read using the Axxin reader. The insulin levels detected by the colorimetric LFD in blood samples are lowest at fasting which increases after breakfast and lunch (Fig. 25). When the detection levels were validated using ELISA, a coefficient of variance (CV) of 0.85 was found.

**[00242] Application of fluorescent based LFDs of Example 2 for detection of insulin levels in human saliva and blood samples**

[00243] To evaluate the practicability and accuracy of the fluorescent based LFD test strips, saliva and blood samples (n=3) (EC number HC190300) were collected (70  $\mu$ L) at different intervals of the day i.e. fasting (over-night fasting), after breakfast (from about 6am to about 10am), after lunch (from about 12pm to about 2pm) and after dinner (from about 6pm to about 8pm) and insulin levels were determined on the LFD at about 30 minutes after each meal. These samples were mixed with Texas red labelled aptamer and incubated at room temperature for 25 minutes. The samples were added to the test strips and incubated for 12 minutes. The results were observed in 12 minutes using the Ax-2x lateral flow reader (Fig. 26). The insulin levels in saliva and blood samples were determined using fluorescent based LFDs and the human insulin ELISA kit. It was observed that the correlation coefficient was 85 % for saliva samples (Fig. 26A) and 86 % for blood samples (Fig. 26B).

**[00244] Calibration curve using human insulin ELISA kit**

[00245] The Wash Buffer Concentrate (25X) was allowed to reach room temperature and was then gently mixed to ensure that any precipitated salts have redissolved before diluting. The Wash Buffer Concentrate (25X) (1 volume) was diluted with 24 volumes of deionized water. All other solutions were prepared accordingly, and the ELISA was carried out according to the protocol. The readings were taken at 450 nm and calibration curve was plotted (see Fig. 27). It was observed that optical density increases linearly with increase in insulin concentration ranging from 0.1 to 1 ng/mL with a linear coefficient of 0.9869. The limit of detection was 0.1 ng/mL, whereas for colorimetric LFDs, this was 0.01 ng/mL (Fig. 20) and for fluorescent based LFDs, this was also 0.01 ng/mL (Fig. 21). This indicates that the LFDs are more sensitive than commercially available human insulin ELISA kit.

**[00246] Validation of colorimetric and fluorescent based LFDs of Example 2 using human insulin ELISA kit**

[00247] Different concentrations (0.1, 0.3, 0.5, 0.7 and 1 ng/mL) of insulin were spiked into PBS (A), serum (B), saliva (C) and blood (D) and the insulin concentrations were detected using colorimetric LFD (Fig. 28A to 28D), fluorescent based LFD (Fig. 29A to 29D) and ELISA. It was found that LFDs had a correlation of about 88-90 % which indicates that the LFDs are a very sensitive tool for insulin detection.

**[00248] Comparison of Smartphone App with the commercially available Axxin reader using colorimetric LFDs of Example 2**

[00249] Different concentrations (0.01, 0.05, 0.1, 0.5 and 1 ng/mL) of insulin were spiked into PBS buffer (0.01 M, pH 7.4) and detected using either the Ax-2x lateral flow reader (Axxin, Victoria, Australia) or the Smartphone App (see Fig. 29). It was found that the readings had a correlation of about 75 % (Fig. 30).

**[00250] RCA-based LFD**

[00251] Rolling Circle Amplification (RCA) for colorimetric analysis was applied to a LFD. Herein, we provide a RCA-based LFD for the detection of insulin in biological samples (an embodiment of which is depicted in Fig. 11).

[00252] The insulin probe was prepared by mixing graphene oxides (GO) with insulin aptamer labelled with FAM (5'-FAM-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3'). Streptavidin-AuNPs and the anti-FAM mAb were pre-immobilized on the conjugate pad and test line of a LFD, respectively. Biotins were pre-immobilized on the control line modified with bovine serum albumin (BSA). The sample pad was dipped into the insulin probe. In the absence of insulin, the insulin probe migrated via capillary action and passed onto the conjugate pad. The insulin probe then rehydrated the streptavidin-AuNP conjugates which

continued to move and was captured on the control line via reactions between biotin and streptavidin on the AuNP surface to form the control line. In this case, no line formed at the test line. In the presence of insulin, the aptamer-FAM dissociated from GO due to high affinity between insulin and the aptamer. The released aptamer-FAM were captured on the test line through specific reactions with the anti-FAM mAb on the test line. The excess streptavidin-AuNP conjugates continued to move and were captured on the control line. Thereafter the RCA reagent containing circular DNA template (5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3'), phi 29 DNA polymerase ( $\phi$ 29DP) and TMB for colorimetric readout, were added to the conjugation pad. The aptamer-FAM fixed at the test line specifically triggered the RCA reaction when the RCA reagent arrived at the test line. The triggered RCA reaction generated DNAzyme which resulted in the colorimetric change of TMB to form a visualized signal.

[00253] Alternatively, the circular DNA template (5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3') is also capable of inducing a fluorescent readout with the aid of SYBR Green II before analysis. When the RCA reagent contained 5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3' and SYBR Green II, it provides a fluorescent readout.

[00254] **Example of Experimental Procedures for RCA-based LFD**

[00255] **Preparation of GO and insulin FAM-aptamer conjugates**

[00256] 1 mg/mL graphene oxide (GO) was ultrasonically pre-treated for 60 minutes, and then diluted into 150  $\mu$ g/ml with PBS. Equal volumes of insulin aptamer ( $C_f$  = 600 nM) were mixed with the GO suspension ( $C_f$  = 0.1 mg/ mL) (1:1 volume ratio) and incubated at room temperature for 30 minutes. The mixture was then blocked with 1% BSA and incubated at room temperature for another 30 minutes. The mixture was then spun down at 14,000 rpm for 5 minutes at 4°C. The supernatant was then removed, and the GO-Aptamer complex was resuspended to the original 600 nM concentration in PBS buffer.

[00257] **Preparation for RCA**

[00258] Circular DNA templates were synthesized from 5'-phosphorylated linear DNA oligonucleotides through template-assisted ligation using T4 DNA ligase. 200 pmol of circular template was first mixed with 10 U PNK and 1 mM ATP in 100  $\mu$ L of 1x PNK buffer A. The mixture was then incubated at 37 °C for 40 minutes followed by heating at 90 °C for 5 minutes. 300 pmol of ligation template was then added and heated at 90 °C for 5 minutes and cooled at room temperature for 15 minutes. Next, 15  $\mu$ L of 10x T4 DNA ligase buffer and 1  $\mu$ L of 10 U T4 DNA ligase was added to the above mixture (total 150  $\mu$ L). The resultant mixture was then incubated at room temperature for 2 h before heating at 90 °C for 5 minutes to deactivate the ligase. The

ligated circular DNA products were concentrated by standard ethanol precipitation and purified by 10% dPAGE.

[00259] After preparing the circular DNA template, the master mix of one single RCA reaction was prepared as follows: 1 pmol of ligated circular DNA, 1  $\mu$ L of 10 mM dNTPs, 0.3  $\mu$ L of 10 U  $\phi$ 29DP, 1  $\mu$ L of 100  $\mu$ M hemin and 5  $\mu$ L of 10% (w/v) pullulan solution.

[00260] **Preparation of RCA-based LFD**

[00261] The sample pad was treated using sample treatment buffer (0.01 M PBS buffer (pH 7.4) containing 1 % BSA, 0.25 % Tween 20, and 2 % sucrose) and incubated at 37°C for 2 hours. While drying, the insulin probe (GO and aptamer conjugates) were used to treat conjugation pad and incubated at 37°C for 1 hour. Streptavidin-AuNPs and the anti-FAM mAb were pre-immobilized on the conjugate pad and test line of the LFD, respectively. Biotins were pre-immobilized on the control line modified with bovine serum albumin (BSA). The sample pad and conjugation pad were stored in a desiccator until needed. Nitrocellulose membrane (NC membrane), sample pad, conjugation pad and absorbent pad were pasted on backing card with 2 mm overlapping each other. Then 4.0 mm wide strips were cut using Strip Guillotine Cutter ZQ2002. After running through the insulin samples, the RCA reagent containing circular DNA template (5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3'),  $\phi$ 29 DNA polymerase ( $\phi$ 29DP) and TMB for colorimetric readout, were dropped down to the conjugation pad. The aptamer-FAM fixed at the test line were able to specifically trigger the RCA reaction when RCA reagent arrived at the test line. The triggered RCA reaction generated DNAzyme resulting in the colorimetric change of TMB to form a visualized signal.

[00262] **Detection of insulin using RCA-based LFD**

[00263] 50  $\mu$ L of insulin sample was dropped onto the sample pad to react with the insulin probe in the conjugation pad at room temperature for 10 minutes. After running through the sample, the RCA reagent was dropped down to the conjugation pad and incubated at room temperature for about 15 minutes to about 30 minutes. The aptamer-FAM fixed at the test line were able to specifically trigger the RCA reaction when RCA reagent arrived at the test line. The triggered RCA reaction generated DNAzyme resulting in the colorimetric change of TMB to form a visualized signal. The intensity of the T-line was quantified by the imageJ software. To obtain a calibration curve, PBS containing different concentrations of insulin (i.e. 0, 0.01, 0.1, 0.2, 0.5, 1, 3, 5, 10, 20, 50, 100, and 200 ng/ mL) were prepared and used as the insulin sample. The performance of the RCA-based LFD was compared and evaluated against the conventional ELISA kit.

[00264] The sensitivity of the RCA-based LFD was increased by 2 folds with a detection limit of 0.001 ng/mL (**Fig. 14**). The RCA-based LFD may be used to target the detection of insulin in exhaled breath condensate.

[00265] **Liposome signal amplification-based LFD with glucose meter readout**

[00266] A liposome signal amplification-based LFD may use a glucose meter (GM) for the detection of insulin (as depicted in Fig. 12). Firstly, glucose loaded liposome are prepared (GLL). The insulin aptamer conjugated to GLL is loaded onto the conjugate pad. The DNA 1 is adsorbed into the test line on the LFD. As the sample moves along the device, the resulting complex is trapped on the test line. The trapped complex is then cut off from the test line, followed by lysing of the liposome to release the glucose encapsulated in the liposome core. The released glucose is then added onto a glucose strip and measured using a personal GM. A liposome signal amplification-based LFD provides enhanced signal compared to an LFD using colloidal gold as probes.

[00267] **Example of Experimental Procedures for liposome signal amplification-based LFD**

[00268] **Preparation of aptamer labelled MNP (Ap-MNP)**

[00269] Aptamer labelled MNP (Ap-MNP) may be prepared by the following protocol. 100 $\mu$ L of the Biotin labelled aptamer (50  $\mu$ M, 5'-biotin-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC) are mixed with 100 $\mu$ L Streptavidin-modified magnetic beads (~50 mg/ mL, 200 nm) dispersed in Tris-HCl buffer solution (100 mM, pH 7.4). The resulting mixture is gently shaking in a sealed centrifugal tube for 6 h at 4 °C on a shaker to encourage biotin and oligonucleotides to conjugate to the streptavidin on magnetic beads. The solution is washed 3 times with 50  $\mu$ L of 10 mM PBS (pH 7.2) with 0.1 M NaCl and 1.0 mM MgCl<sub>2</sub>. The product is collected after magnetic separation to purify the achieved aptamer-magnetic beads. The mixture is characterised using Dynamic light scattering (DLS, Zetasizer) to see the difference before and after conjugation and confirm the formation of conjugates. The aptamer-functionalized magnetic beads are incubated with 3.0wt% BSA at room temperature for 60 minutes to block the unreacted active sites.

[00270] **Preparation of glucose loaded liposomes (GLL)**

[00271] GLL may be prepared by the following protocol. The water bath is put into the fume hood and set at 45 °C. A solvent is prepared including chloroform and methanol in the fume hood (10 mL; 3:1 v/v). A mixture of 125 mg of HSPC, 25 mg of cholesterol, and 5 mg of PEA (molar ratio 50:10:1) are dissolved in the mixed solvent. The resulting mixture is sonicated (Qsonica 432B Sonicator Sonabox Sound Ecclosure) at 45 °C in a water bath under nitrogen for 30 minutes until a homogeneous mixture is formed. A 5.0 mL, 0.1 M glucose solution is prepared in deionized

water and is kept in the 45°C water bath for 10 minutes. The glucose solution (5.0 mL, 0.1 M) is injected into the mixture using a syringe. The mixture is sonicated for another 5 minutes at 45°C to reduce the sizes of the GLLs to about 200 nm. The resulting solution is kept in a water bath at 45°C for 2 hours in the dark (turn off the light of the fume hood and wrap the container with aluminium foil) to remove the organic solvents. The GLLs are extruded using a 0.4 µm polycarbonate filter. The process is repeated for at least 10 times to produce a homogeneous suspension of uniformly sized GLLs. The suspension is stored at 4 °C when not in use. The dispersion of GLLs are separated by using a column chromatography method (e.g. use a Sephadex-G 50 column (20 cm × 2.5 cm)) and the mixture is washed with PBS (pH 7.4) at a flow rate of 0.5 ml/min.

**[00272] Conjugation of complementary DNA (cDNA) to glucose loaded liposomes (aptamer-GLL)**

[00273] Conjugation of cDNA to GLL may be prepared by the following protocol. 2.0 mL of the cDNA (5'-COOH-GAAGACACCCTAC-3') which is complementary to the insulin aptamer above is mixed with Tris-HCl buffer (100 mM, pH 7.4) containing EDC (2.5 mg/mL) and NHS (2.5 mg/mL). The mixture is allowed to react for 20 minutes at room temperature to activate the carboxyl group on the cDNA. The mixture is incubated with 2.0 mL of the glucose-loaded liposomes for 6 hours at room temperature. During the incubation period, the cDNA is covalently connected to GLLs. The GLLs are collected using centrifugation (13,000 rpm) at 4°C and the unreacted cDNA in the supernatant is removed. The conjugates are dispersed in 5.0 mL of 100 mM Tris-HCl buffer (pH 7.4).

**[00274] Preparation of insulin probes MNP-Ap-cDNA-GLLs**

[00275] Insulin probes MNP-Ap-cDNA-GLLs may be prepared by the following protocol. 5 µL of the cDNA-GLLs and 5 µL of the Ap-MNP are mixed and are incubated in Tris-HCl buffer solutions for 100 minutes at 37°C to form MNP-Ap-cDNA-GLLs. The MNP-Ap-cDNA-GLLs are collected using magnetic separation in order to remove unreacted cDNA-GLLs.

**[00276] Preparation of liposome signal amplification-based LFD**

[00277] A LFD may be prepared by the following protocol. The sample pad is treated using sample treatment buffer (0.01 M PBS buffer (pH 7.4) containing 1 % BSA, 0.25 % Tween 20, and 2 % sucrose) and is incubated at 37°C for 2 hours. While drying, 5 mL of insulin probe (MNP-Ap-cDNA-GLLs) is introduced onto the conjugate pad. The middle portion of the NC membrane is used as a test line which is first fixed with streptavidin followed by the attachment of biotin-aptamer (5'-biotin-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC). The strips are cut to 4 mm in width. All the pre-treated pads are assembled onto a backing card. Sample solutions

containing the different concentrations of insulin are added to the loading pad and allowed to migrate through the entire strip by capillary action, specifically binding to the Ap-MNP to release the cDNA-GLLs due to competitive binding. The released cDNA-GLLs will rest on the test line. The complex that will form on the test line is cut off and 100  $\mu$ L of 10 mg/mL Triton X-100 is added to help release the encapsulated glucose. A 5.0  $\mu$ L aliquot of the resulting solution containing glucose molecules released from liposomes is dropped onto the glucose test strip and then detected by a GM.

We claim:

1. A kit for detecting insulin in a sample comprising
  - (A) an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component; and
  - (B) a lateral flow assay device (LFD) comprising
    - (i) a sample region; and
    - (ii) a detection region comprising
      - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
      - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe.
2. The kit of claim 1, wherein the LFD further comprises a conjugation pad and/or an absorbent pad.
3. The kit of claim 1 or claim 2, wherein the aptamer specific for insulin comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
4. The kit of any one of claims 1 to 3, wherein the control component comprises a sequence of 5'-TTT TTT TTT TTT TTT TTT TT-3' and the detection component comprises gold nanoparticles (AuNPs).
5. The kit of any one of claims 1 to 3, wherein the control component comprises a sequence of 5'-TTT TTT TTT TTT TTT TTT TT-3' and the detection component comprises a fluorescence dye.
6. The kit of claim 5, wherein the fluorescence dye is Texas red.
7. The kit of any one of claims 1 to 6, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TT-3'.
8. The kit of any one of claims 1 to 7, wherein the control component binding molecule comprises a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'.
9. The kit of any one of claims 1 to 8, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3'.

10. A kit for detecting insulin in a sample comprising:
- (A) an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise gold nanoparticles (AuNPs);
- and
- (B) a lateral flow assay devise (LFD) comprising
    - (i) a sample region;
    - (ii) a conjugation pad;and
    - (iii) a detection region comprising
      - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprises a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
      - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3'.
11. A kit for detecting insulin in a sample comprising:
- (A) an insulin probe comprising an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprises the fluorescence dye Texas red; and
  - (B) a lateral flow assay devise (LFD) comprising
    - (i) a sample region;
    - (ii) a conjugation pad;and
    - (ii) a detection region comprising
      - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprises a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
      - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3'.

12. The kits of any one of claims 1 to 11, wherein the lateral flow assay device further comprises an absorbent pad.
13. The kit of any one of claims 1 to 12, wherein the control component binding molecule is immobilized on the control line by streptavidin-biotin affinity reaction.
14. The kit of any one of claims 1 to 13, wherein the aptamer binding molecule specific for the aptamer is immobilized on the test line by streptavidin-biotin affinity reaction.
15. The kit of any one of claims 1 to 14, wherein the control component binding molecule comprised on the control line and the molecule specific for the aptamer comprised on the test line are present at a ratio of 1:3.
16. A lateral flow assay device (LFD) for detecting insulin in a sample comprising
  - (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component; and
  - (ii) a detection region comprising
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
    - (b) a test line comprising an aptamer binding molecule specific for the aptamer comprised in the insulin probe.
17. A lateral flow assay device (LFD) for detecting insulin in a sample comprising
  - (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component; and
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe; and
    - (b) a test line comprising an aptamer binding molecule specific for the aptamer comprised in the insulin probe.
18. A lateral flow assay device (LFD) for detecting insulin in a sample comprising
  - (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component,

wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise gold nanoparticles (AuNPs);

and

- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprises a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3'.

19. A lateral flow assay device (LFD) for detecting insulin in a sample comprising

- (i) a sample region;
- (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a control component, and a detection component, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC TTT TTT TTT TTT TTT TTT TT-3', and wherein the detection component comprise the fluorescence dye Texas red;

and

- (iii) a detection region comprising
  - (a) a control line comprising a control component binding molecule specific for the control component comprised in the insulin probe, wherein the control component binding molecule comprising a sequence of 5'-AAA AAA AAA AAA AAA AAA AA-3'; and
  - (b) a test line comprising a molecule specific for the aptamer comprised in the insulin probe, wherein the molecule specific for the aptamer comprises a sequence of 5'-GAA GAC ACC CTA CCA ACC CCC CCC ACC ACC-3'.

20. The LFD of any one of claims 16 to 19 further comprising an absorbent pad.

21. The LFD of any one of claims 16 to 20, wherein the control component binding molecule is immobilized to the control line by streptavidin-biotin affinity reaction.

22. The LFD of any one of claims 16 to 21, wherein the molecule specific for the aptamer is immobilized to the test line by streptavidin-biotin affinity reaction.

23. The LFD of any one of claims 16 to 22, wherein the control component binding molecule comprised on the control line and the molecule specific for the aptamer comprised on the test line are present at a ratio of 1:3.
24. A method for detecting insulin comprising the steps of
- (a) applying a sample to the sample region of the LFD defined in any one of claims 16 to 23;
  - (b) incubating the LFD; and
  - (c) assessing the intensity of the test line.
25. The method of claim 24, wherein the LFD is incubated for about 2 minutes to about 15 minutes.
26. The method of claim 24, wherein the LFD is incubated for about 10 to about 15 minutes.
27. The method of any one of claims 24 to 26, wherein the method has a detection limit of about 0.01 ng/mL.
28. A kit for detecting insulin from a sample comprising
- (A) an insulin probe comprising an aptamer specific for insulin, a target component, and an RCA capture sequence;
  - (B) graphene oxide (GO);
  - (C) Rolling Circle Amplification (RCA) reaction mixture comprising
    - (i) a circular RCA template;
    - (ii) a mix of dNTPs; and
    - (iii) a detection component;
  - (D) a DNA polymerase;
- and
- (E) a lateral flow assay device (LFD) comprising
    - (i) a sample region;
    - (ii) a conjugation pad comprising a control component;and
    - (iii) a detection region comprising
      - (a) a control line comprising a control component binding molecule; and
      - (b) a test line comprising a target component binding molecule.
29. The kit of claim 28, wherein the LFD further comprises an absorbent pad.

30. The kit of claim 28 or claim 29, wherein the insulin probe is provide pre-absorbed on the GO.
31. The kit of any one of claims 28 to 30, wherein the aptamer specific for insulin comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
32. The kit of any one of claims 28 to 31, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3'.
33. The kit of any one of claims 28 to 32, wherein the target component comprises fluorescein (FAM).
34. The kit of any one of claims 28 to 33, wherein the control component comprises streptavidin-AuNPs.
35. The kit of claim 34, wherein the control probe binding molecule comprises biotin-bovine serum albumin (BSA).
36. The kit of any one of claims 28 to 35, wherein the target binding molecule is anti-FAM monoclonal antibody.
37. The kit of any one of claims 28 to 36, wherein the RCA template comprises a sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3'.
38. The kit of any one of claims 28 to 36, wherein the RCA template comprises a sequernce of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3'.
39. The kit of any one of claims 28 to 37, wherein the detection component comprises 3,3',5,5'-tetramethylbenzidine (TMB) and wherein the RCA reaction mixture further comprises hemin.
40. The kit of any one of claims 28 to 36 or 38, wherein the detection component comprises a cyanine dye such as SYBR Green II.
41. A kit for detecting insulin in a biological sample comprising:
  - (A) an insulin probe comprising an aptamer specific for insulin, a target component, and an RCA capture sequence, wherein the insulin probe comprises the sequence of of

5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component comprises fluorescein (FAM);

- (B) graphene oxide (GO);
  - (C) Rolling Circle Amplification (RCA) reaction mixture comprising
    - (i) a circular RCA template comprising a sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC CTACC CAAAA GTCAG ATGAA-3';
    - (ii) a mix of dNTPs;
    - (iii) 3,3',5,5'-tetramethylbenzidine (TMB); and
    - (iv) hemin;
  - (D) phi 29 DNA polymerase;
- and
- (E) a lateral flow assay device (LFD) comprising:
    - (i) a sample region;
    - (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs); and
    - (iii) a detection region comprising:
      - (a) a control line comprising a control component binding molecule, wherein the control component comprises biotin-bovine serum albumin (BSA); and
      - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody.

42. A kit for detecting insulin in a biological sample comprising:

- (A) an insulin probe comprising an aptamer specific for insulin, a target component, and a RCA capture sequence, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component comprises fluorescein (FAM);
  - (B) graphene oxide (GO);
  - (C) Rolling Circle Amplification (RCA) reaction mixture comprising
    - (i) a ligated circular RCA template comprising the sequence of 5'-TTGAA GTGAG AAAAC CCAAC CCGCC GTTGG GTTTT GTCAG ATGAA-3';
    - (ii) a mix of dNTPs; and
    - (iii) a cyanine dye such as SYBR Green II;
  - (D) phi 29 DNA polymerase;
- and
- (E) a lateral flow assay device (LFD) comprising:
    - (i) a sample region;

- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs); and
  - (iii) a detection region comprising:
    - (a) a control line comprising a control component binding molecule, wherein the control component comprises biotin-bovine serum albumin (BSA); and
    - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody.
43. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region comprising:
    - (a) an insulin probe comprising an aptamer specific for insulin, a target component, and a capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO);
    - (b) a control component;and
  - (ii) a detection region comprising:
    - (a) a control line comprising a control component binding molecule; and
    - (b) a test line comprising a target component binding molecule.
44. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and a capture sequence; wherein the insulin probe is absorbed onto graphene oxide (GO);
  - (ii) a conjugation pad comprising a control component;
- and
- (iii) a detection region comprising:
    - (a) a control line comprising a control component binding molecule; and
    - (b) a test line comprising a target component binding molecule.
45. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises an aptamer specific for insulin, a target component, and a capture sequence, wherein the insulin probe is absorbed onto graphene oxide (GO); and wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC CTCAC TTCAA TTCAT CTGAC-3', and wherein the target component comprises fluorescein (FAM);

- (ii) a conjugation pad comprising a control component, wherein the control component comprises streptavidin-gold nanoparticles (AuNPs); and
  - (iii) a detection region comprising
    - (a) a control line comprising a control component binding molecule, wherein the control component binding molecule comprises biotin-bovine serum albumin (BSA); and
    - (b) a test line comprising a target component binding molecule, wherein the target component binding molecule comprises anti-FAM monoclonal antibody.
46. The LFD of any one of claims 43 to 45 further comprising an absorbent pad.
47. A method for detecting insulin comprising the steps of:
- (a) adding a sample to the sample region of the LFD defined in any one of claims 43 to 46;
  - (b) incubating the LFD;
  - (c) adding to the detection region of the LFD a Rolling Circle Amplification (RCA) reaction mixture comprising
    - (i) a circular RCA template;
    - (ii) a mixture of dNTPs;
    - (iii) a DNA polymerase; and
    - (iii) a detection component;
  - (d) incubating the LFD;
- and
- (e) assessing the intensity on the test line.
48. The method of claim 47, wherein the LFD is incubated for about 10 to about 15 minutes prior to the addition of RCA mixture.
49. The method of claim 47 or claim 48, wherein the LFD is incubated for about 15 to about 30 minutes after to the addition of RCA mixture.
50. A method for detecting insulin comprising the steps of:
- (a) adding a sample to the sample region of the LFD defined in any one of claims 43 to 46;
  - (b) adding to the sample region of the LFD a Rolling Circle Amplification (RCA) reaction mixture comprising
    - (i) a circular RCA template;
    - (ii) a mixture of dNTPs;

- (iii) a DNA polymerase; and
    - (iii) a detection component;
  - (c) incubating the LFD;
  - and
  - (d) assessing the intensity on the test line.
51. The method of claim 50, wherein the LFD is incubated for about 15 to about 30 minutes after the addition of RCA mixture
52. The method of any one of claims 47 to 51, wherein the detection component comprises 3,3',5,5'-tetramethylbenzidine (TMB) and wherein the RCA reaction mixture further comprises hemin.
53. The method of any one of claims 47 to 51, wherein the detection component comprises a cyanine dye such as SYBR Green II.
54. The method of any one of claims 47 to 53, wherein the method has a detection limit of about 0.001 ng/mL.
55. A kit for detecting insulin in a sample comprising
- (A) an insulin probe comprising a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer;
  - (B) a surfactant;
- and
- (C) a lateral flow assay device (LFD) comprising
    - (i) a sample region; and
    - (ii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the glucose loaded liposome.
56. The kit of claim 55, wherein the lateral flow assay device further comprises an absorbent pad.
57. The kit of claim 55 or claim 56, wherein the GLL-capture molecule comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
58. The kit of any one of claims 55 to 57, wherein the LFD further comprises a conjugation pad and/or an adsorbant pad.

59. The kit of any one of claims 55 to 58, wherein the aptamer specific for insulin comprises a sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
60. The kit of any one of claims 55 to 59, wherein the molecule specific for the first aptamer comprised on the surface of the GLL comprises a sequence of 5'-GAAGACACCCTAC-3'.
61. A kit for detecting insulin from a sample comprising:
- (A) an insulin probe comprising a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises the sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
  - (B) a surfactant;
- and
- (C) a lateral flow assay device (LFD) comprising:
    - (i) a sample region;
    - (ii) a conjugation pad;and
    - (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein GLL-capture molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
62. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer; and
  - (ii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL.
63. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer;
- and

- (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL.
64. A lateral flow assay device (LFD) for detecting insulin in a sample comprising:
- (i) a sample region;
  - (ii) a conjugation pad comprising an insulin probe, wherein the insulin probe comprises a first aptamer specific for insulin bound to a glucose loaded liposome (GLL), wherein the surface of the GLL comprises a molecule specific for the first aptamer, wherein the insulin probe comprises a sequence of 5'-GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3' and a magnetic bead; and wherein the molecule comprised on the surface of the GLL comprises the sequence 5'-COOH-GAA GAC ACC CTA C-3';
- and
- (iii) a detection region comprising a test line comprising a GLL-capture molecule specific for the molecule comprised on the surface of the GLL, wherein the GLL-capture molecule comprises the sequence of 5' GGT GGT GGG GGG GGT TGG TAG GGT GTC TTC-3'.
65. The LFD of any one of claims 62 to 64, further comprising an absorbent pad.
66. A method for detecting insulin comprising the steps of:
- (a) adding a sample to the sample region of a LFD defined in any one of claims 62 to 65;
  - (b) incubating the LFD;
  - (c) isolating the test line comprised in the detection region of the LFD;
  - (d) releasing the glucose from the GLL captured on the test line; and
  - (e) assessing the glucose level released from the GLL.
67. The method of claim 66, wherein the glucose is released from the liposome using a non-ionic surfactant.
68. The method of claim 67, wherein the surfactant is a non-ionic surfactant such as Triton X-100.
69. The method of any one of claims 66 to 68, wherein the glucose level is assessed by a glucose test strip and a portable glucose meter.
70. The method of any one of claims 66 to 69, wherein the LFD is incubated for about 2 minutes to about 15 minutes.

71. The method of any one of claims 66 to 69, wherein the LFD is incubated for about 10 to about 15 minutes.
72. The method of any one of claims 24 to 27, 47 to 54, or 66 to 71, wherein the sample is saliva, serum, blood, urine, or exhaled breath condensate.
73. The method of any one of claims 24 to 27, 47 to 54, or 66 to 71, wherein the sample is saliva.
74. The method of any one of claims 24 to 27, 47 to 54, or 66 to 71, wherein the sample is exhaled breath condensate.
75. The method of any one of claims 24 to 27, 47 to 54, or 66 to 74, wherein insulin is measured in a sample obtained from a subject that has abstained from food and/or beverage for at least about 6 to about 8 hours.
76. The method of any one of claims 24 to 27, 47 to 54, or 66 to 74, wherein insulin is measured in a sample from a subject that has abstained from food and/or beverage for about 2 to about 5 hours.
77. The method of any one of claims 24 to 27, 47 to 54, or 66 to 74, wherein insulin is measured in a sample from a subject that has abstained from food and/or beverage for about for less than about 60 minutes to less than about 1 minute.
78. The method of any one of claims 24 to 27, 47 to 54, or 66 to 77, wherein the insulin is assessed and/or tracked using a smartphone.
79. The method of any one of claims 24 to 27 or 47 to 54, wherein the insulin is assessed using a commercially available lateral flow assay device reader.
80. The lateral flow assay device described in any one of claims 1 to 79, wherein the sample region comprises a sample filter membrane.
81. The lateral flow assay device described in any one of claims 1 to 80, wherein the sample pad and conjugation pad are a single pad.

Figure 1

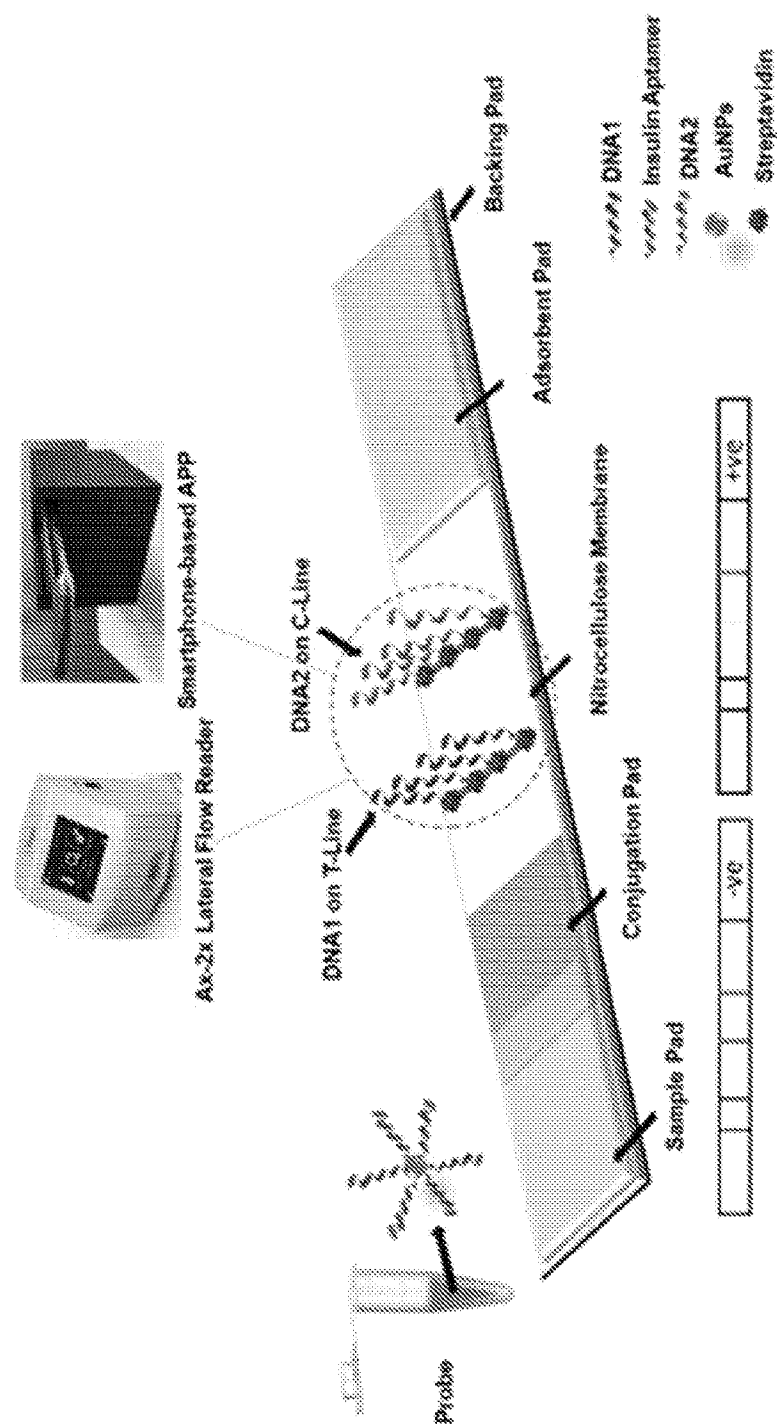
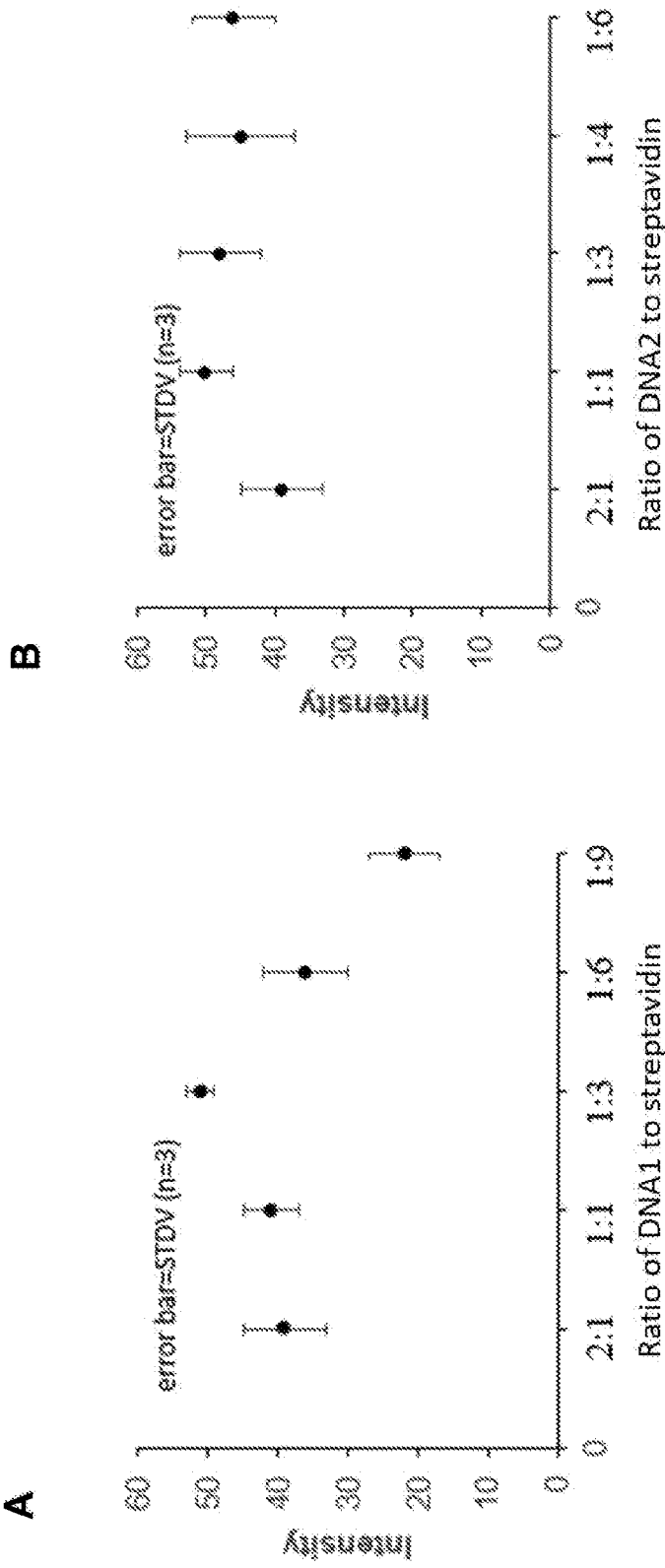
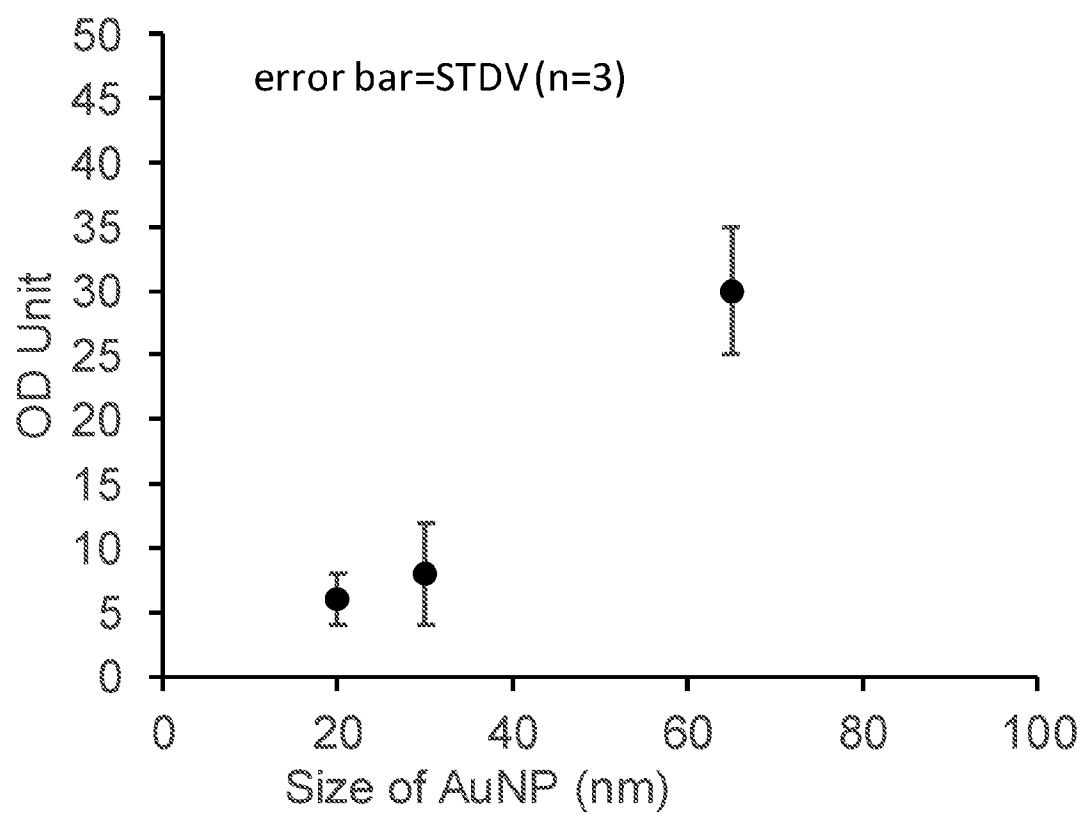
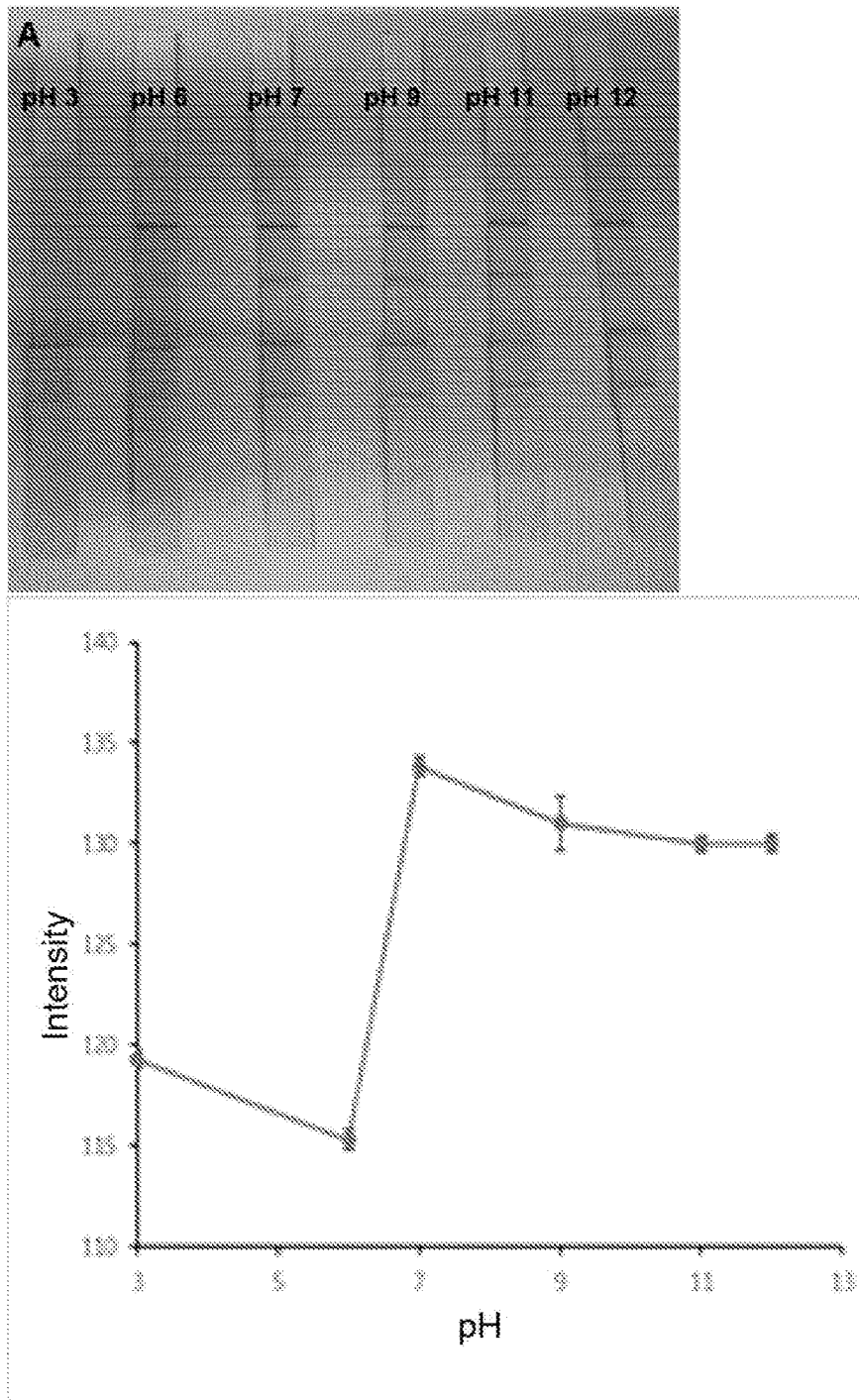
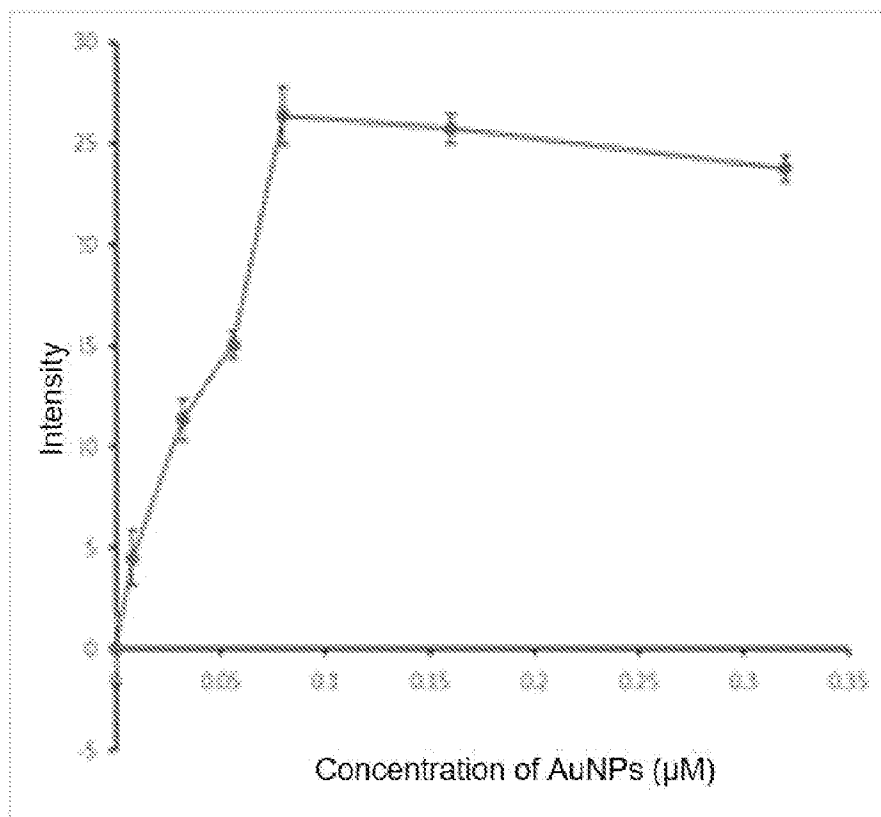
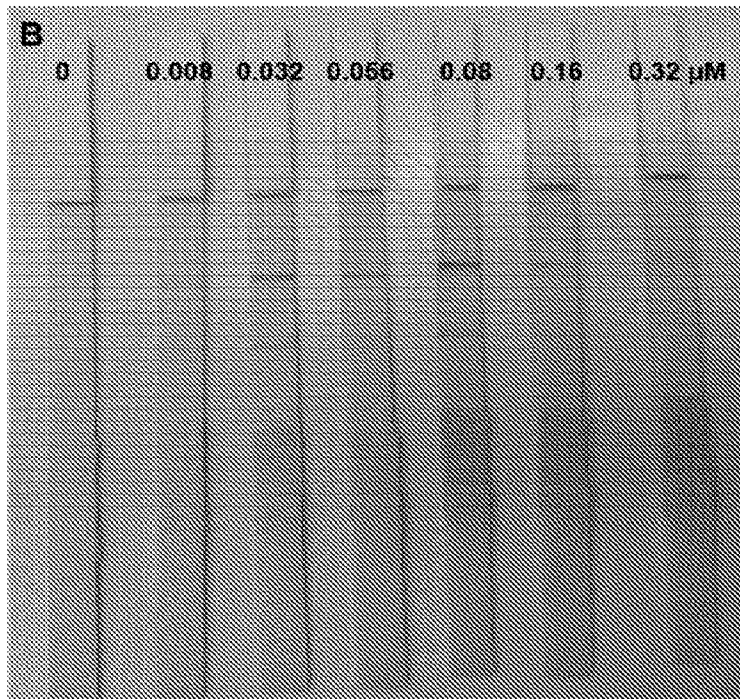


Figure 2



**Figure 3**

**Figure 4**

**Figure 4 continued**

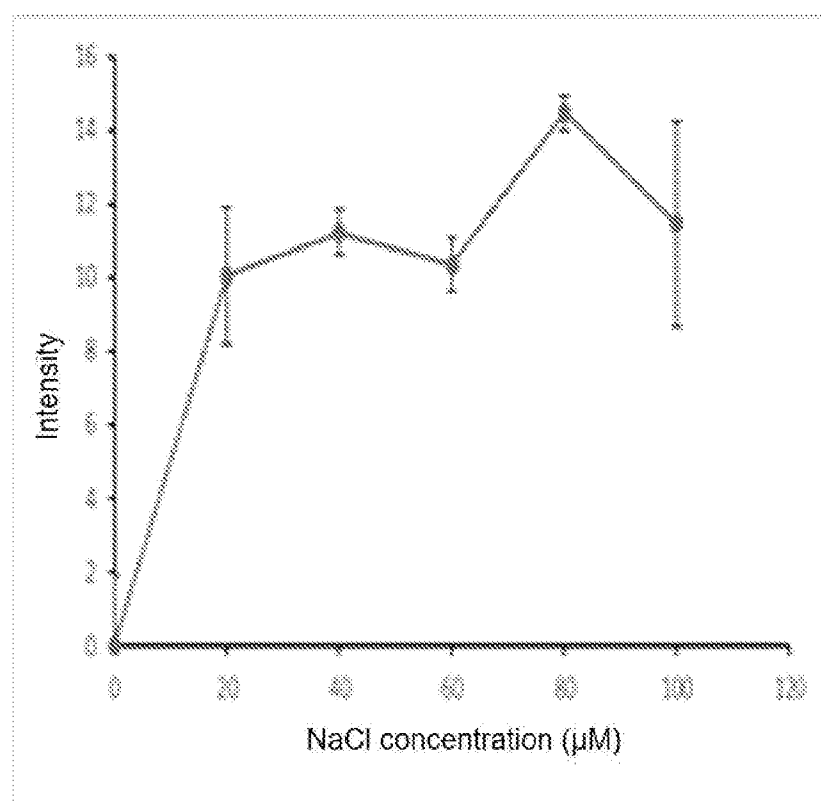
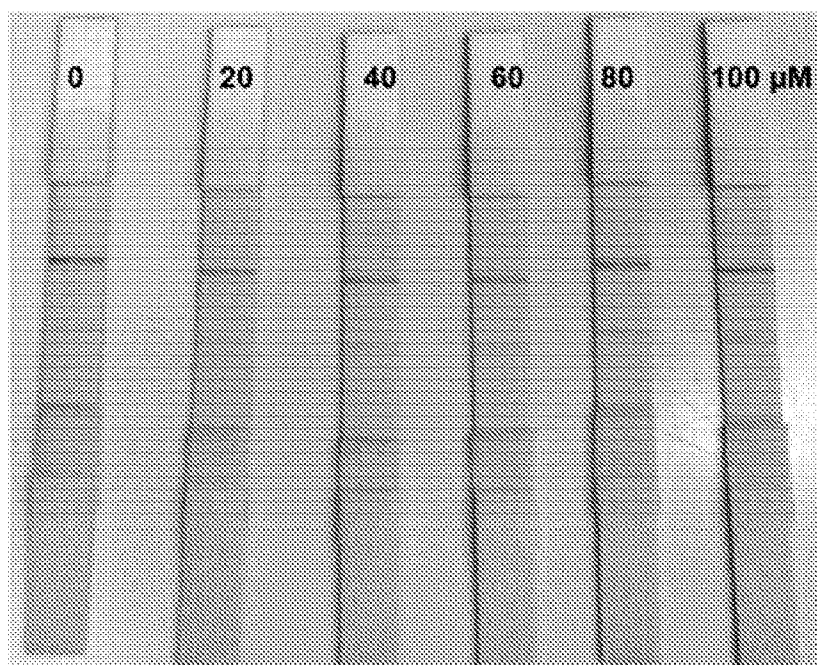
**Figure 5**

Figure 6

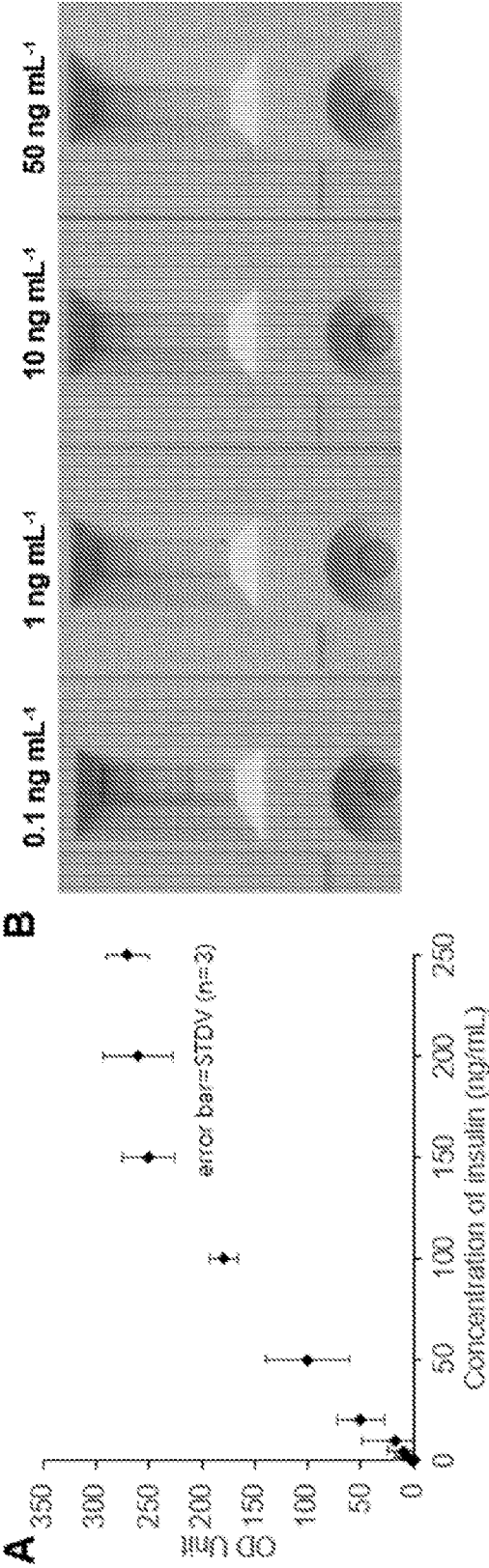


Figure 7

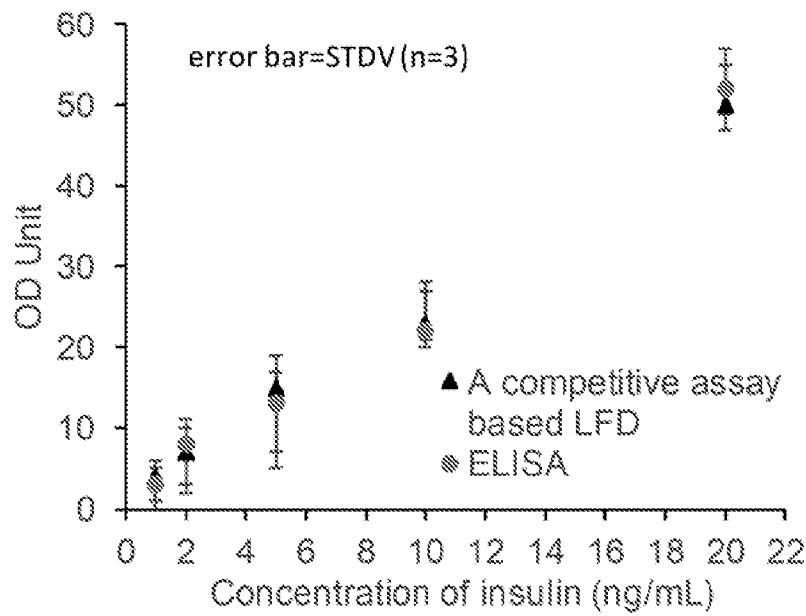


Figure 8

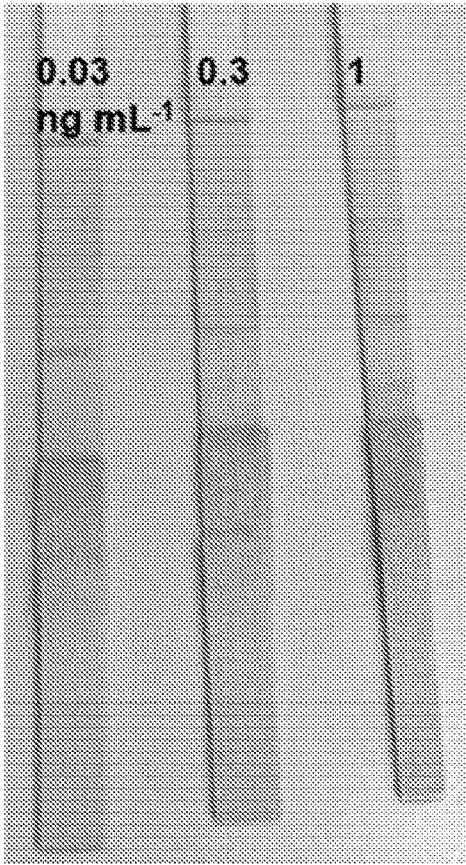


Figure 9

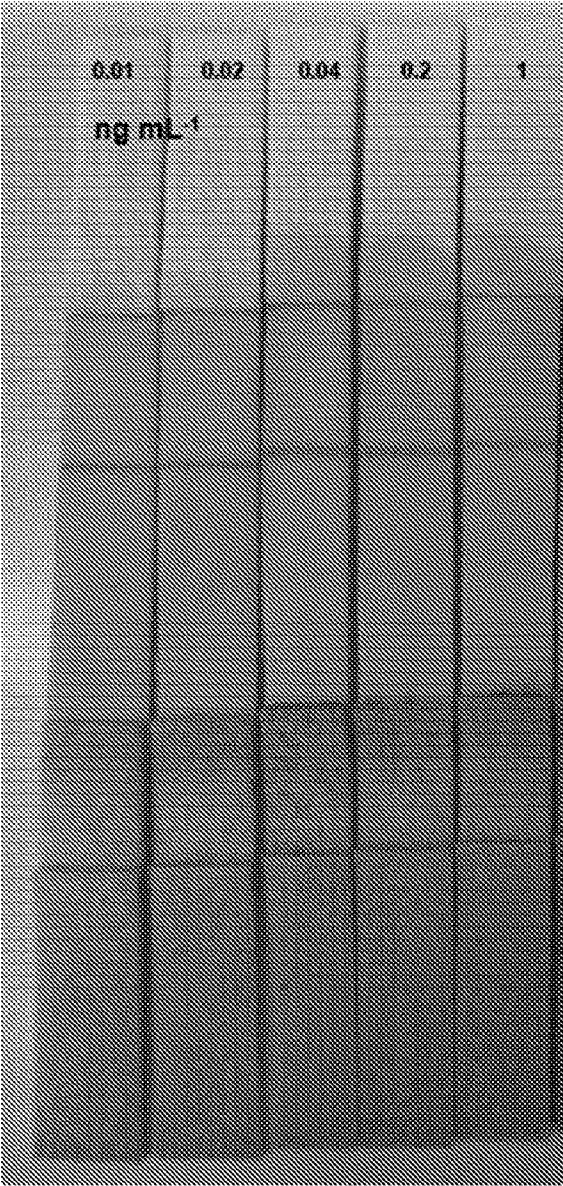


Figure 10

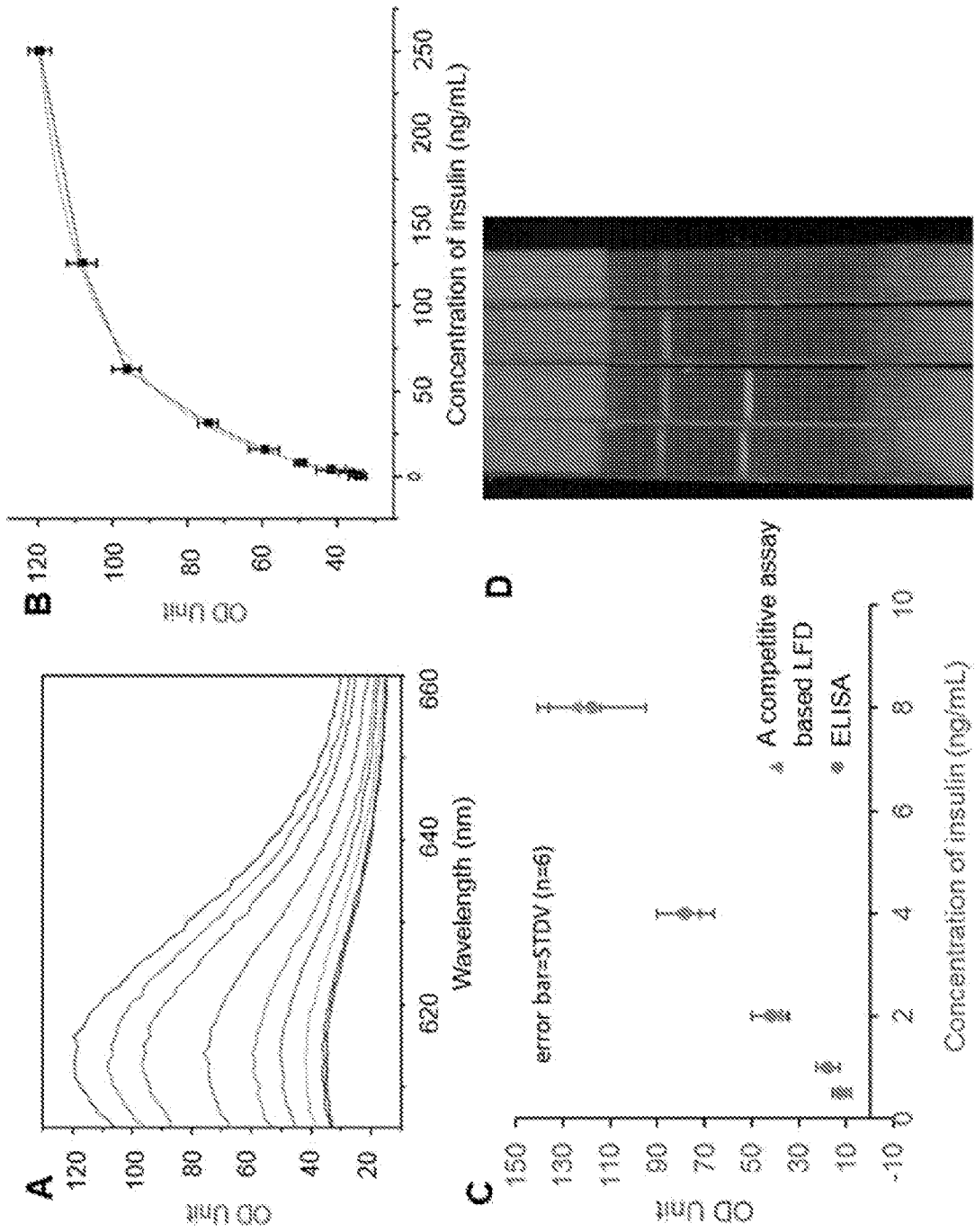


Figure 11

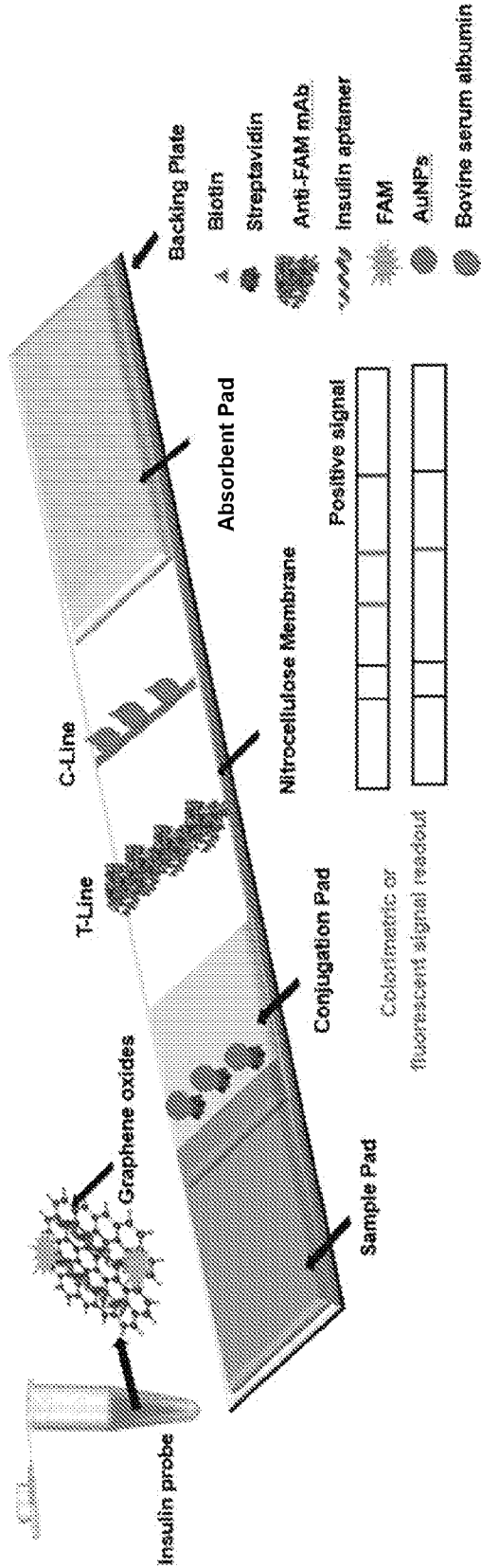


Figure 12

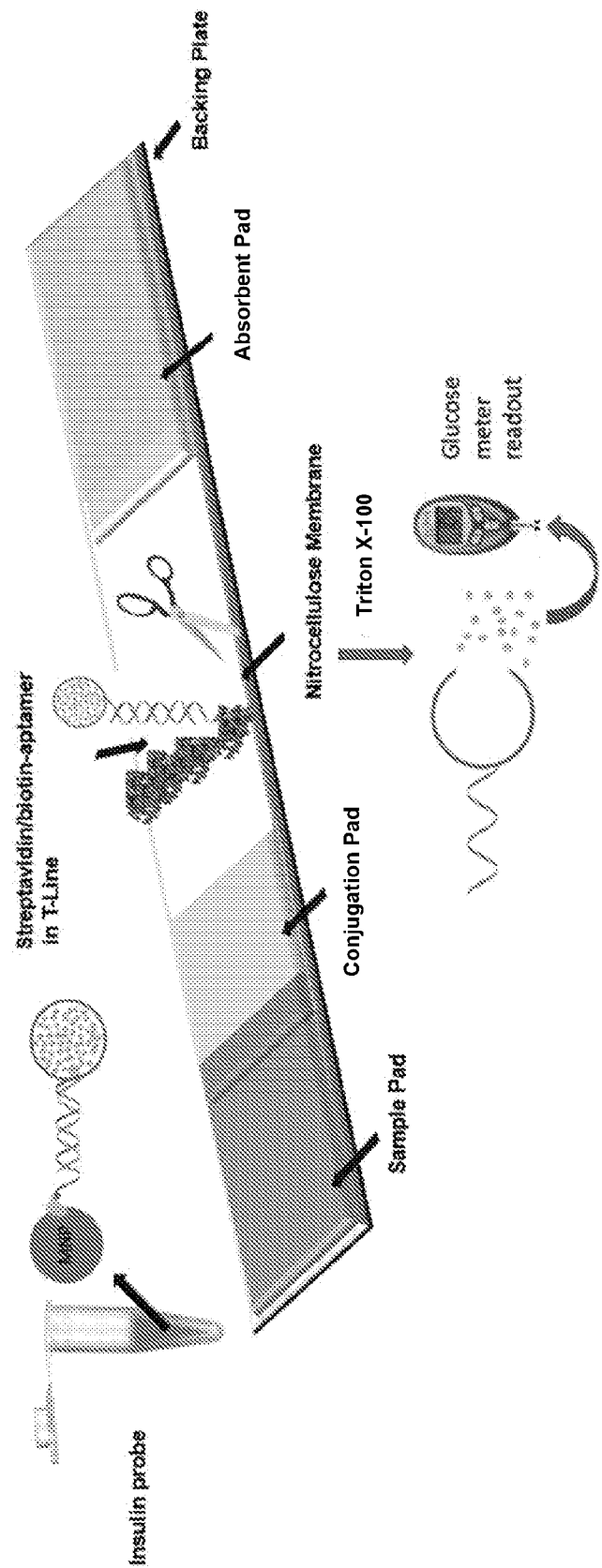


Figure 13

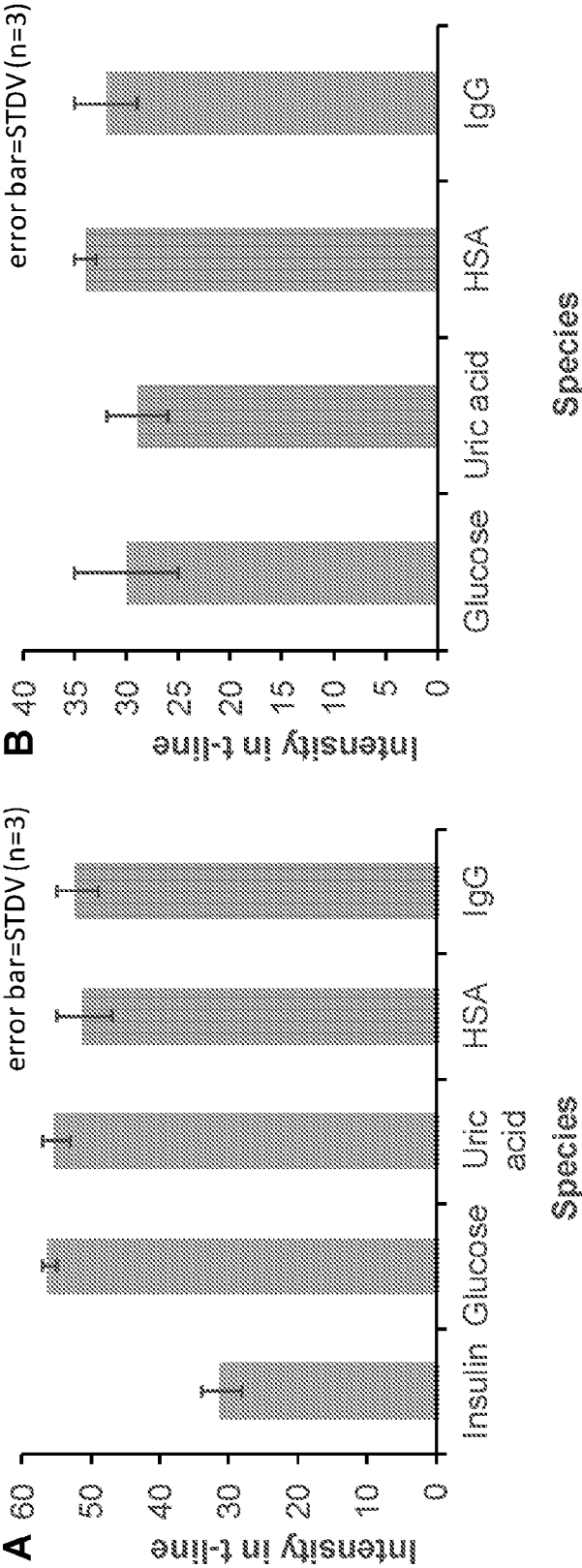
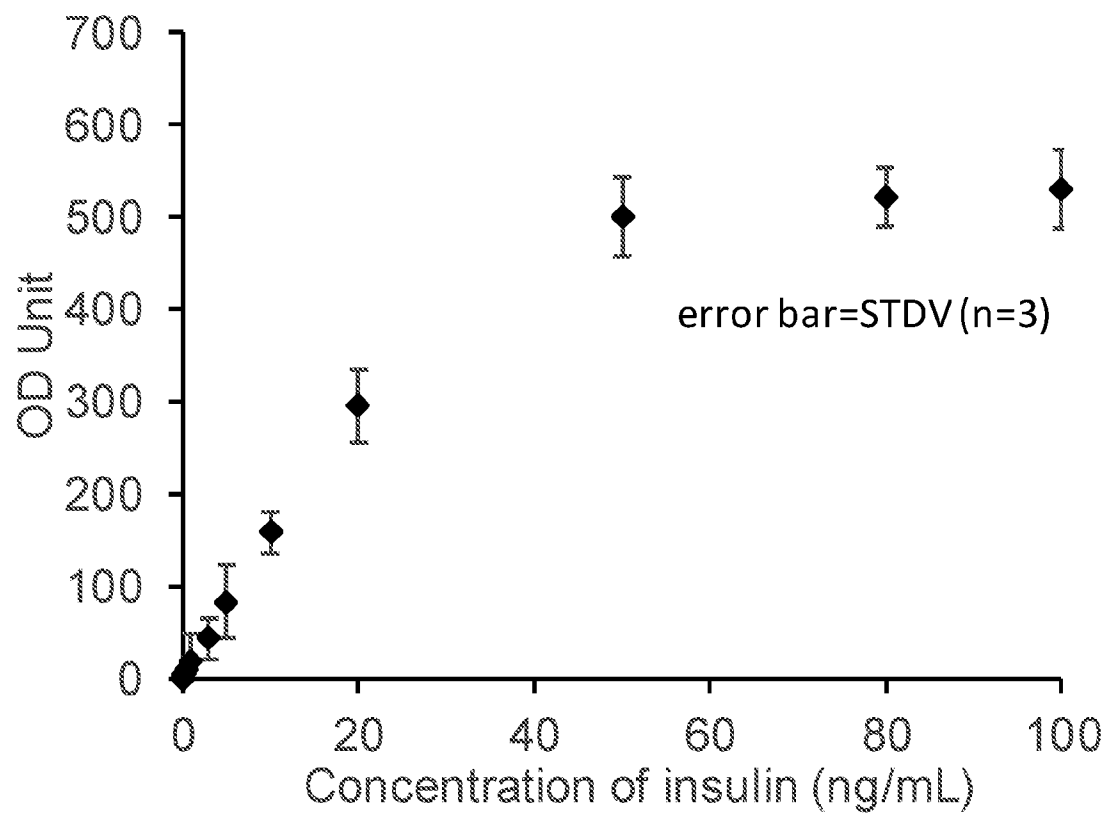
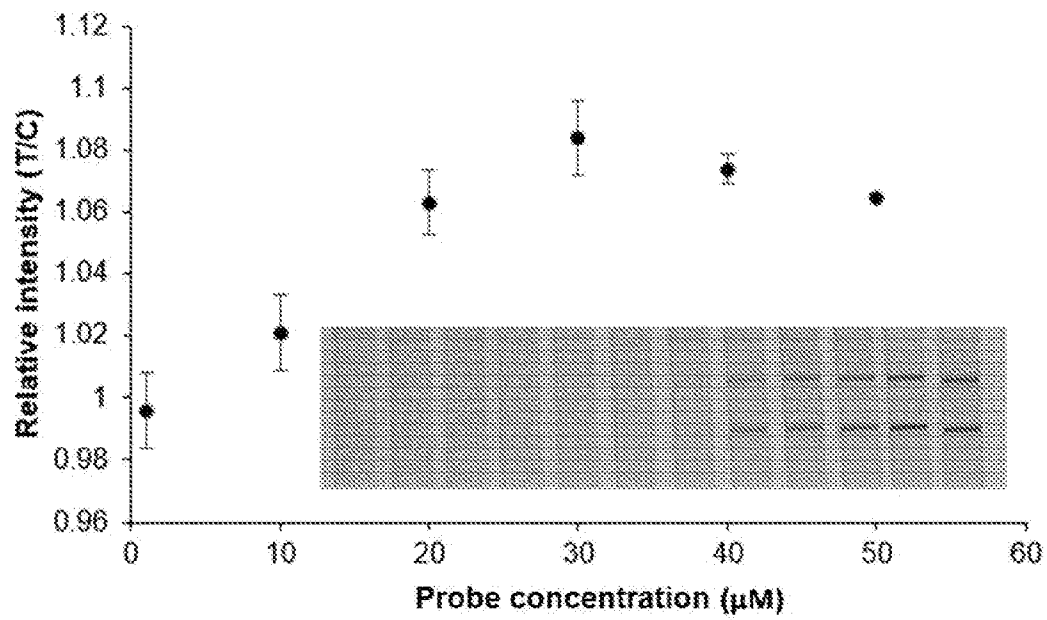
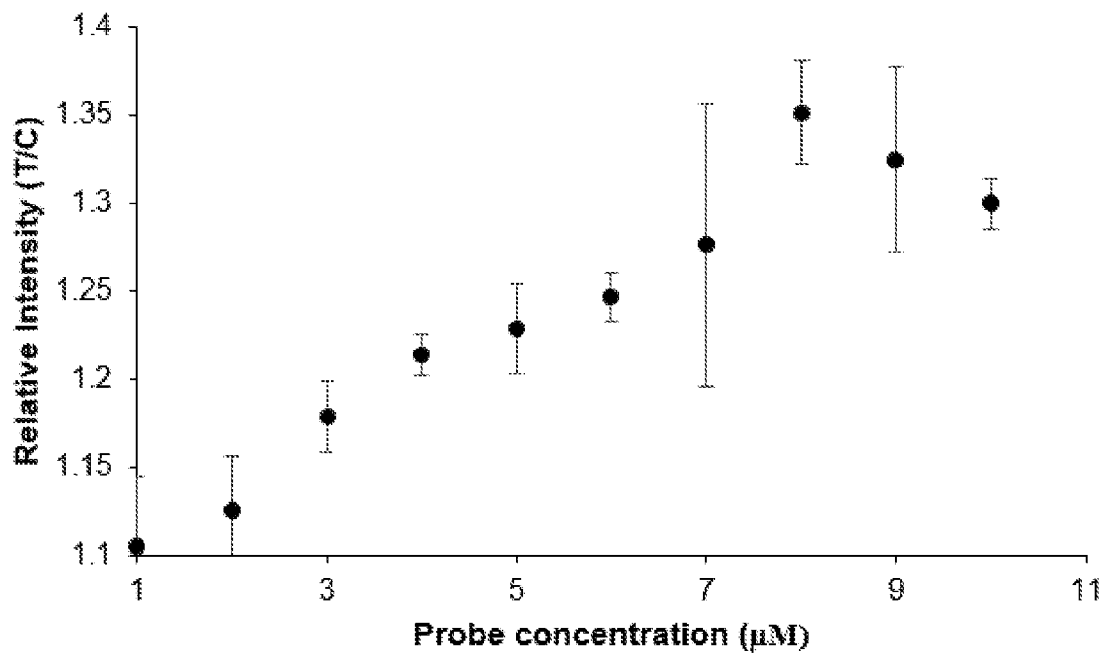
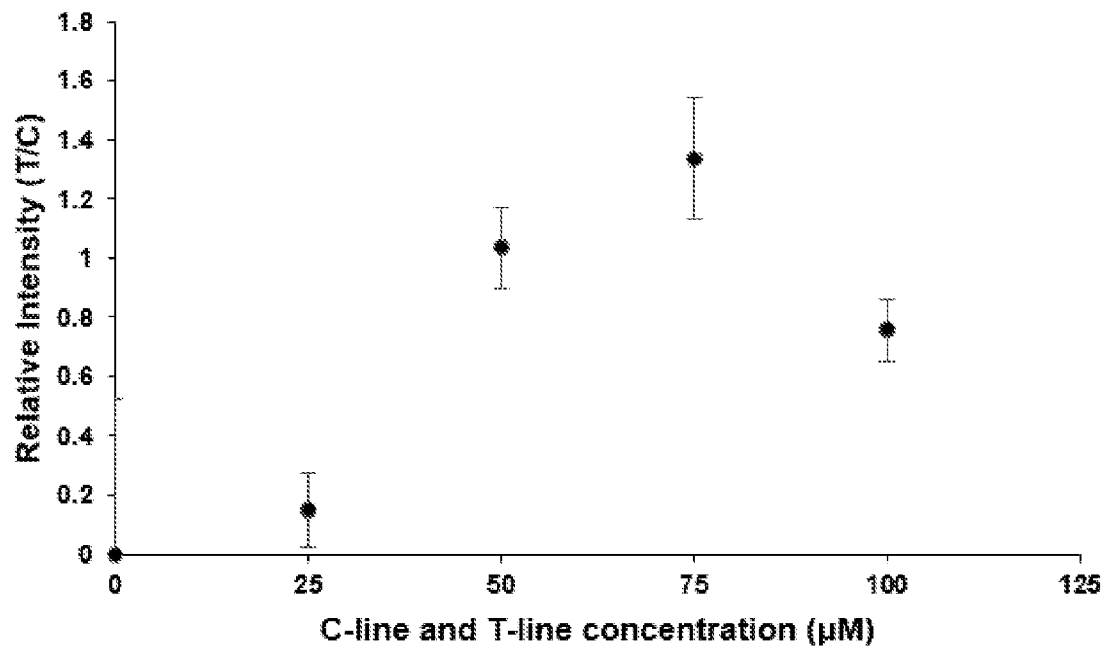
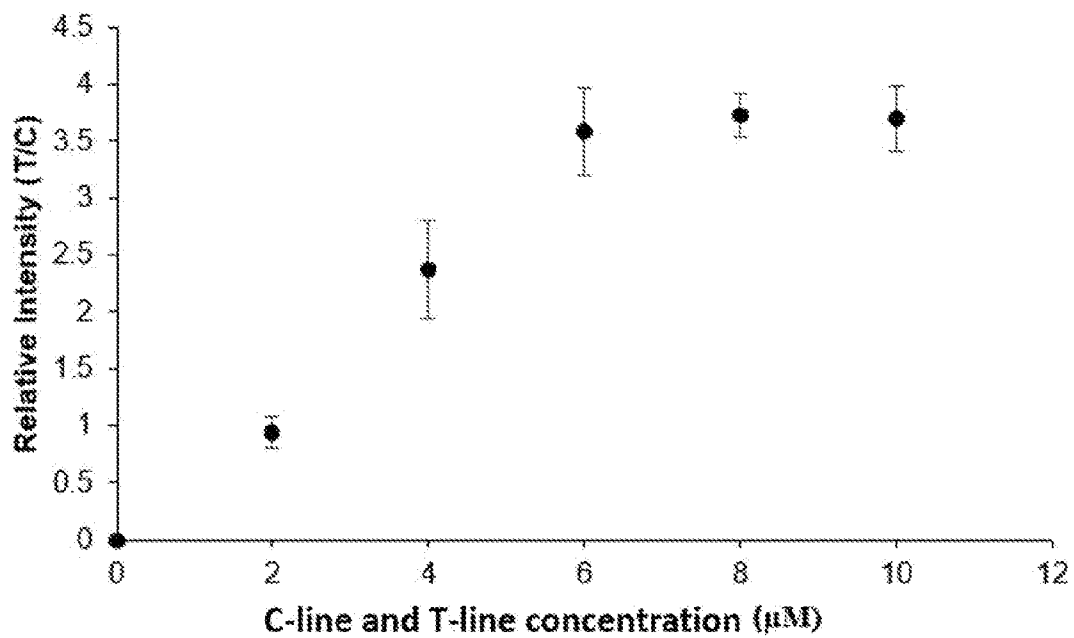


Figure 14



**Figure 15****Figure 16**

**Figure 17****Figure 18**

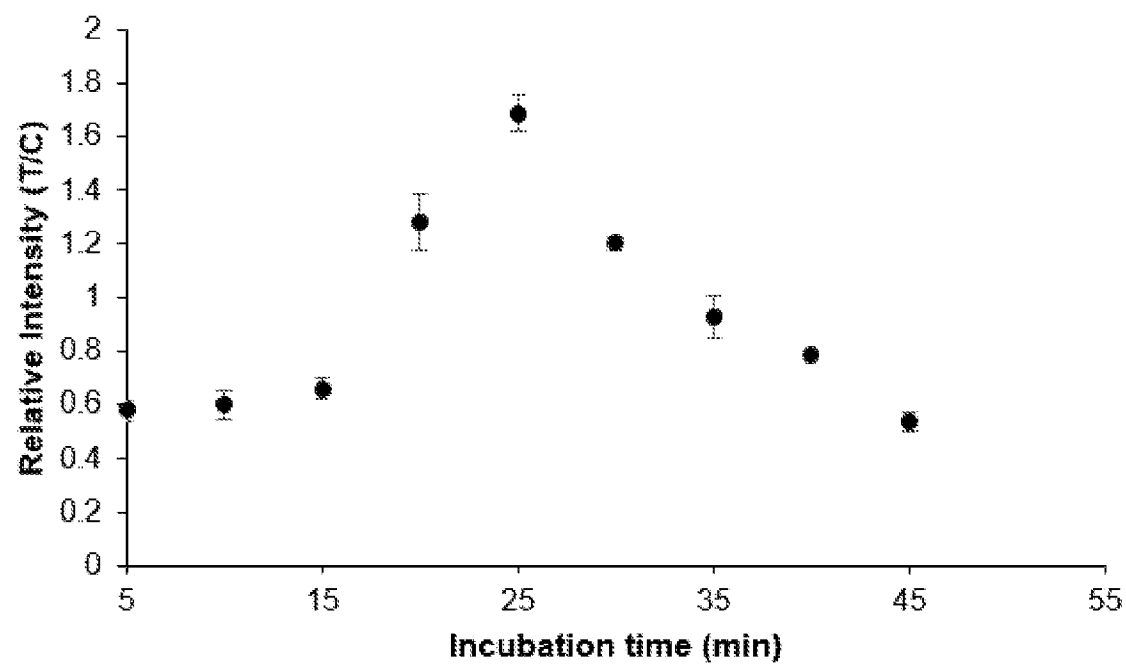
**Figure 19**

Figure 20

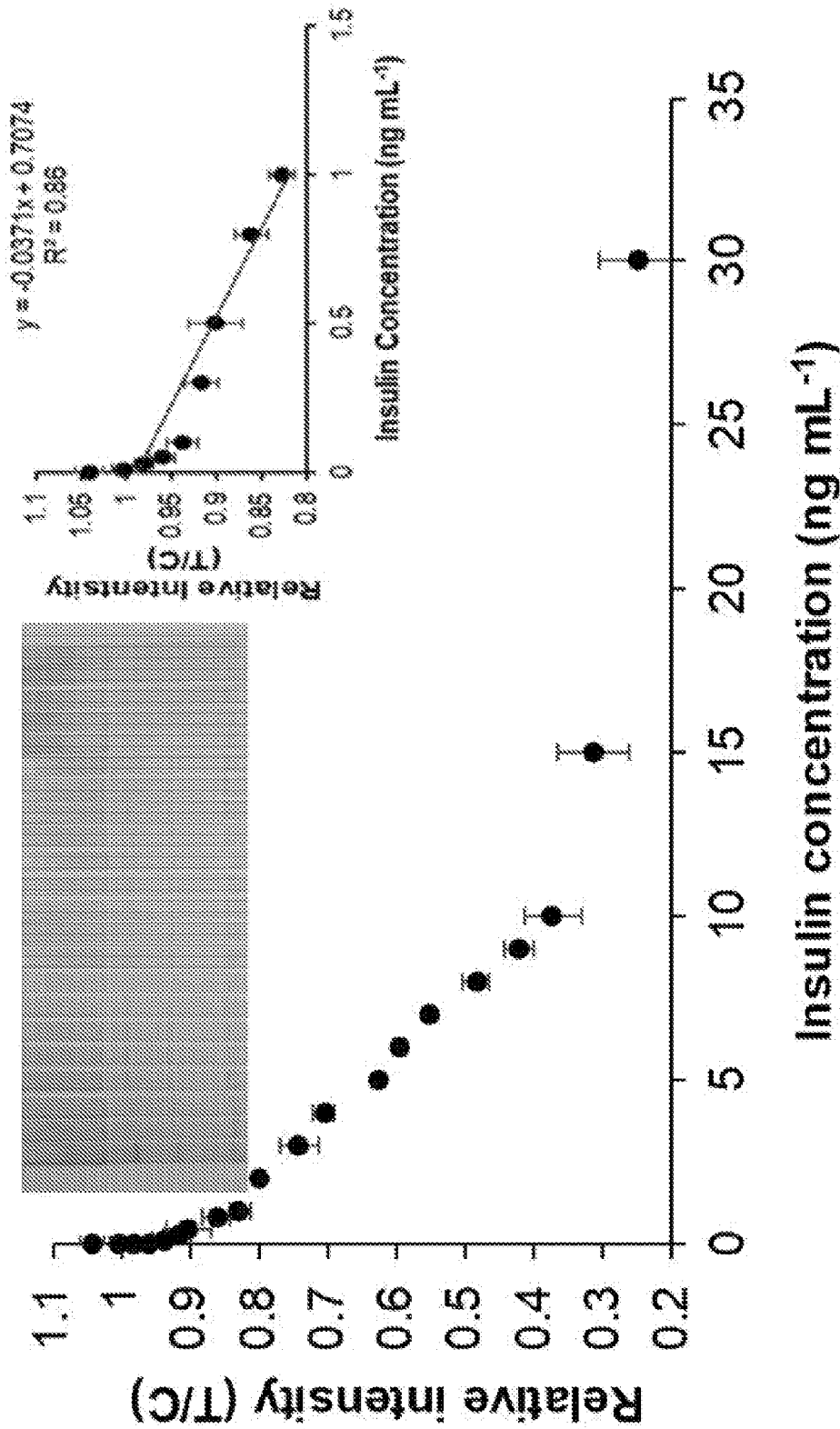
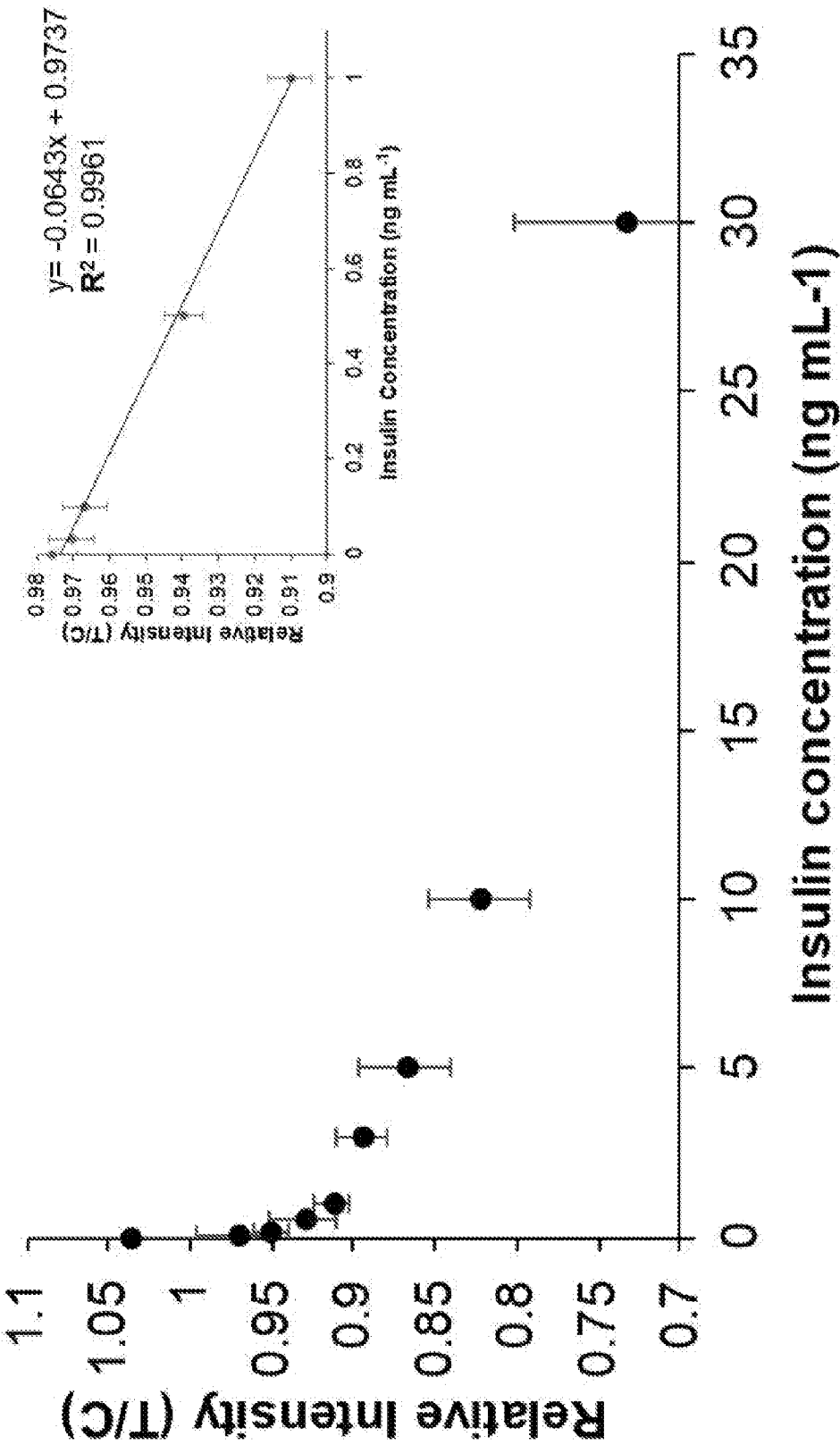


Figure 21



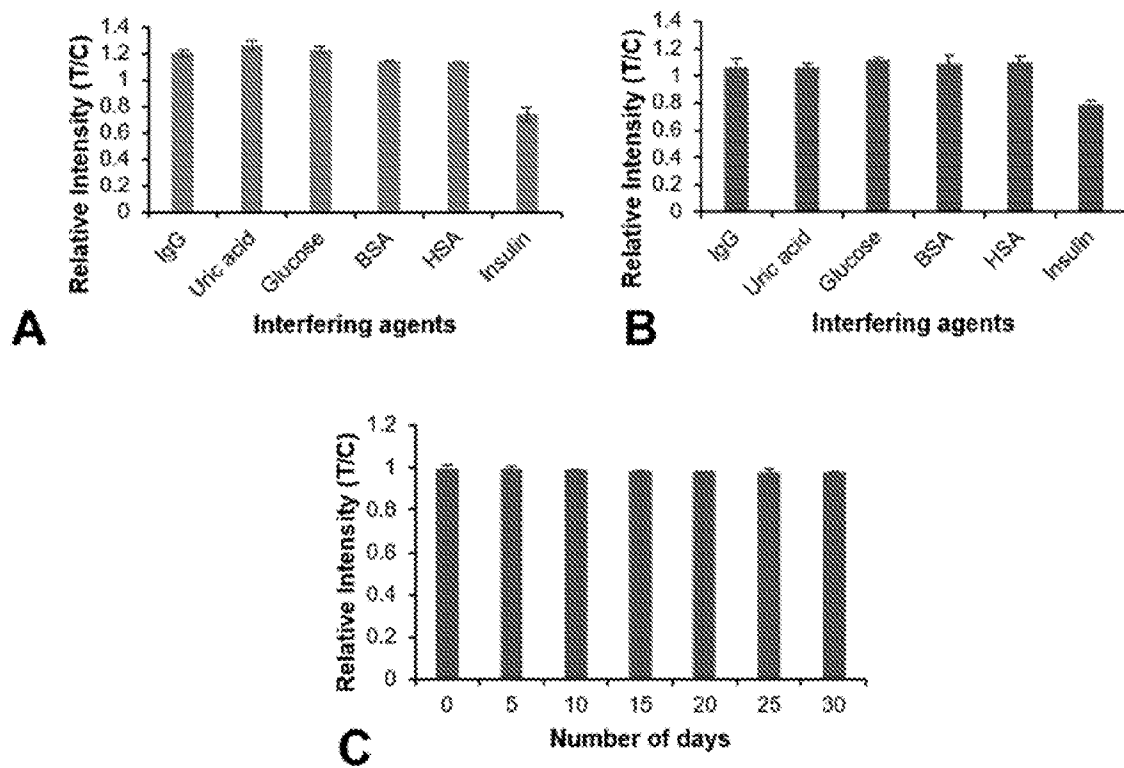
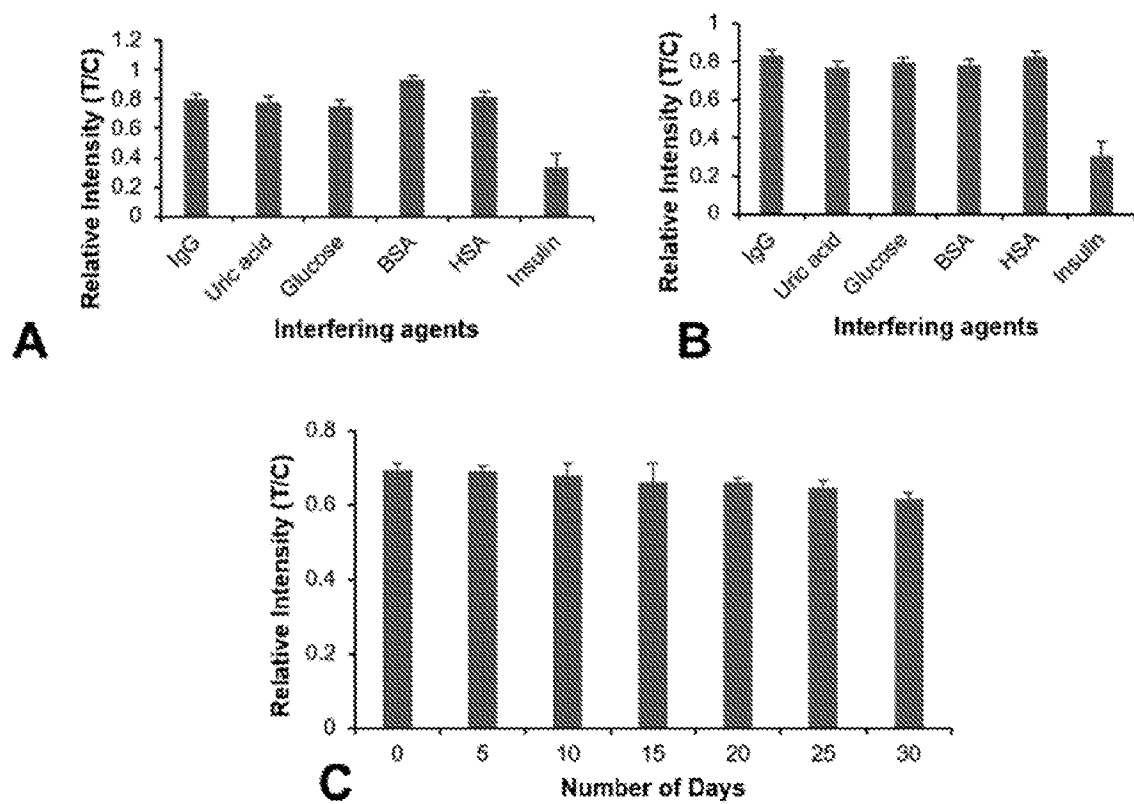
**Figure 22****Figure 23**

Figure 24

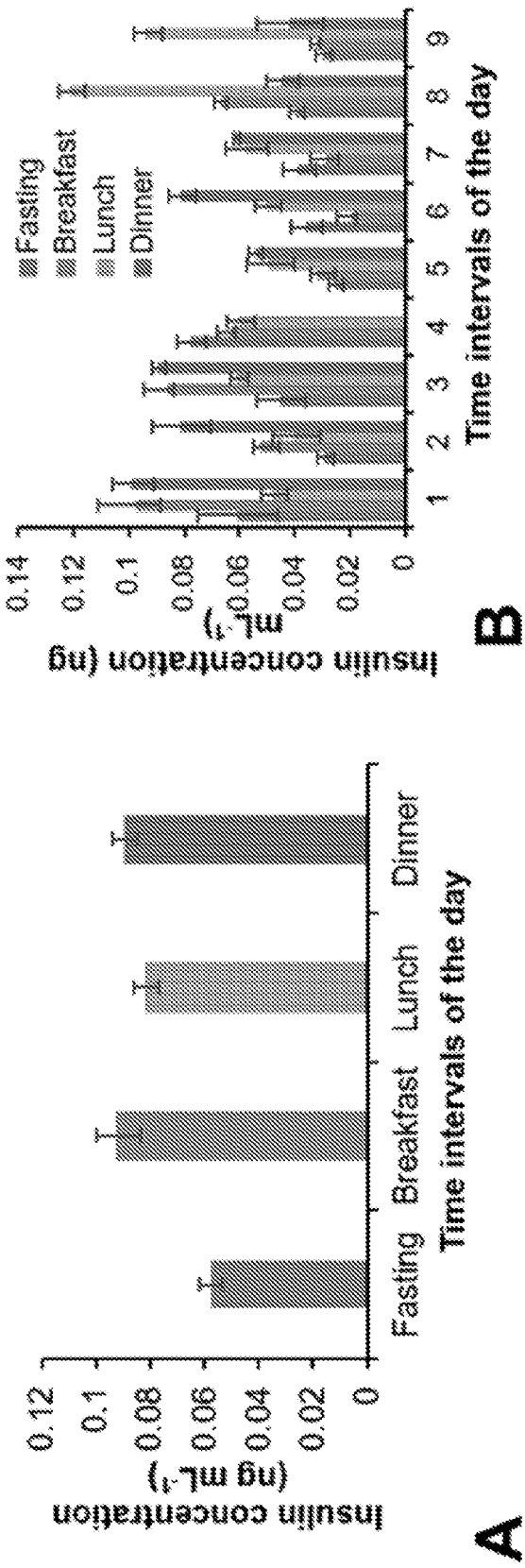


Figure 25

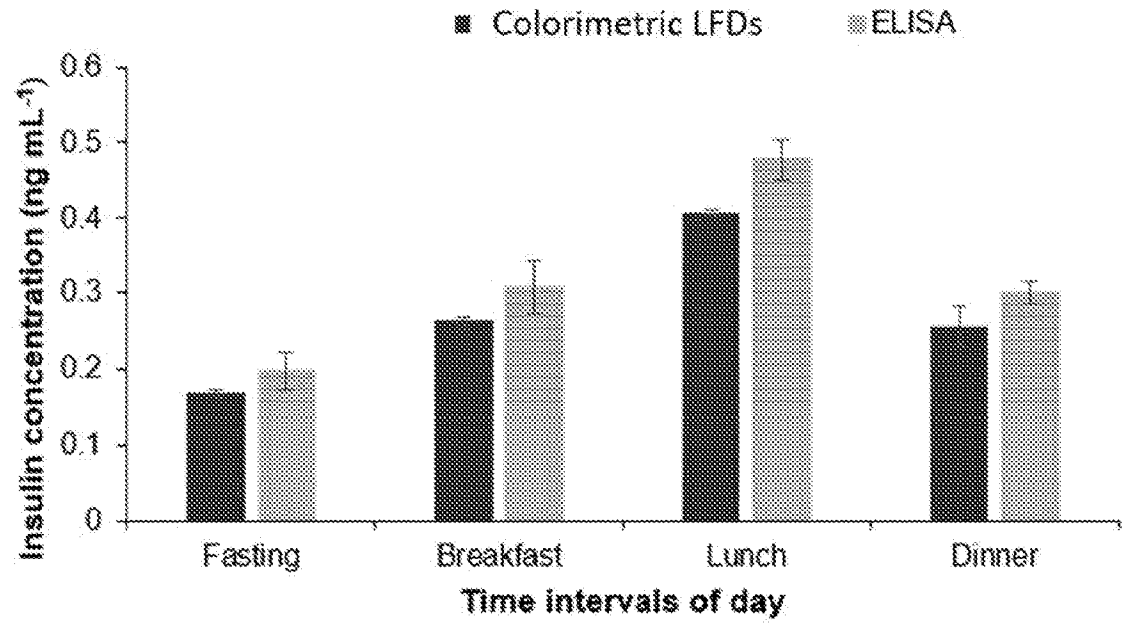
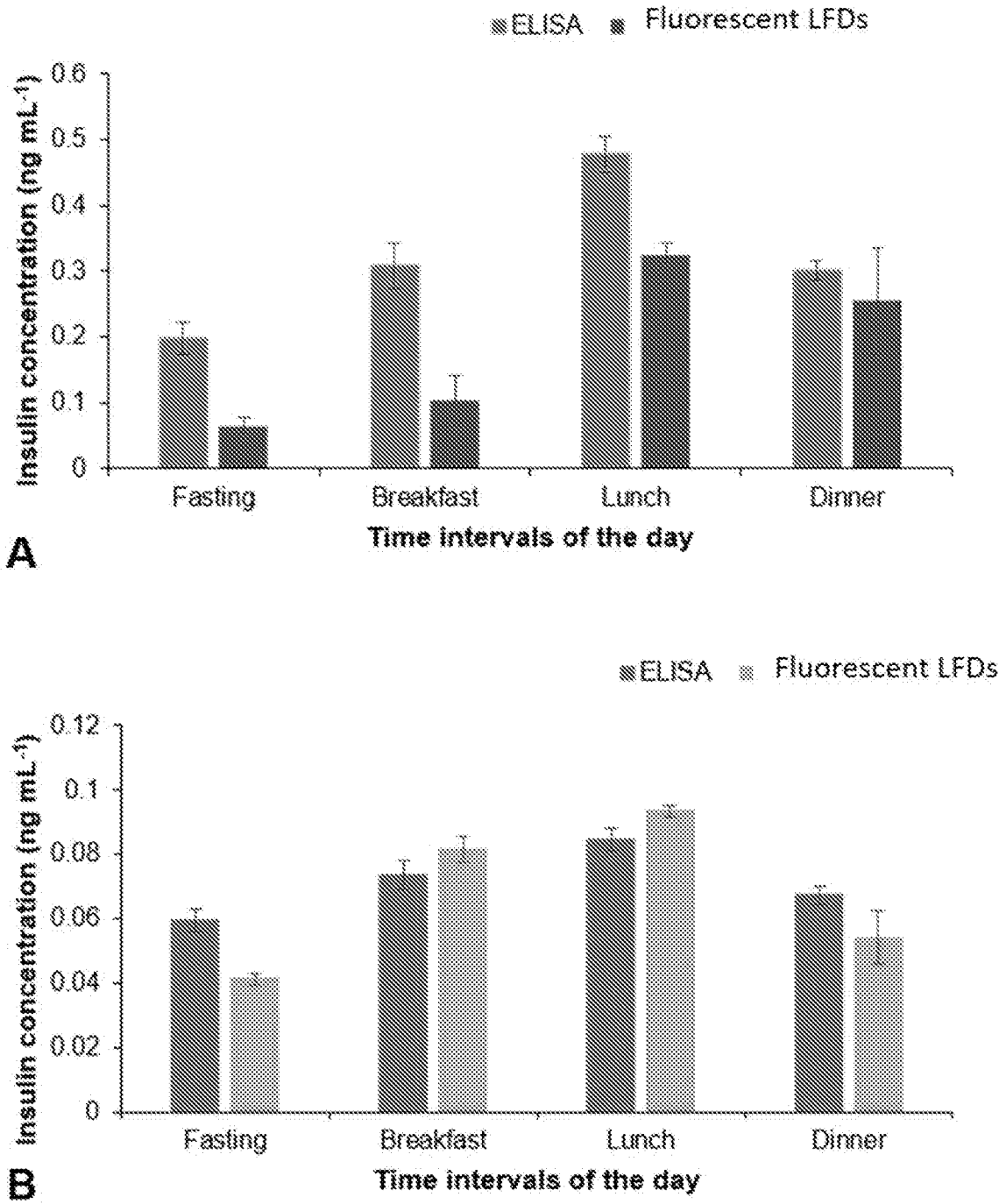
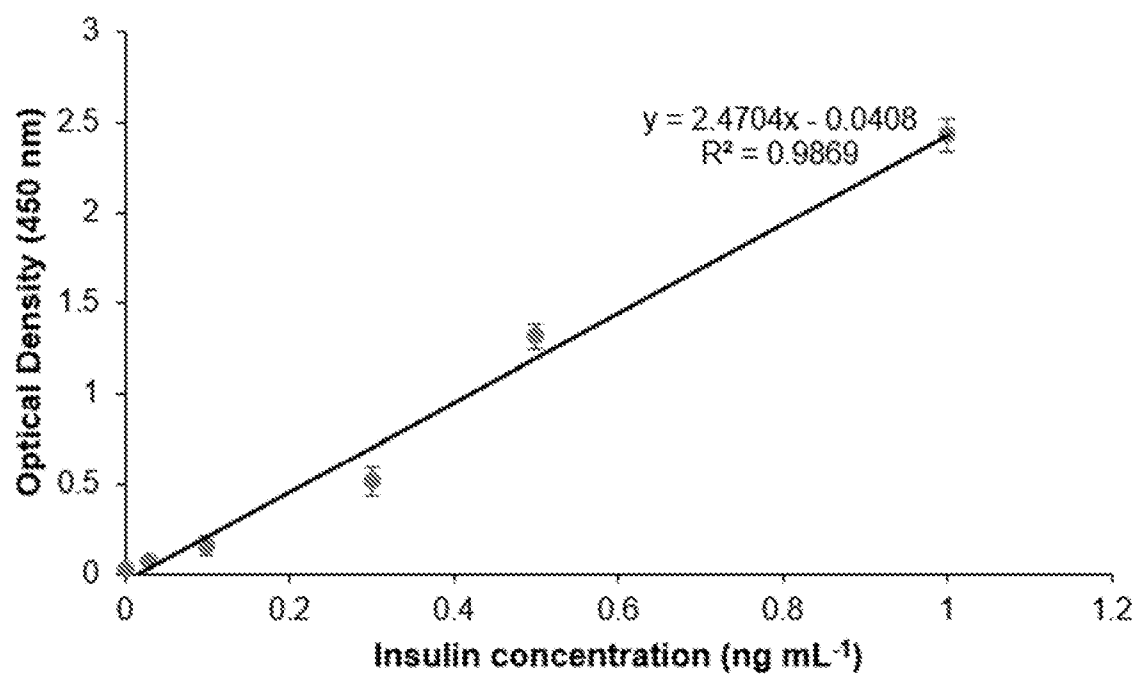


Figure 26



**Figure 27**

## Figure 28

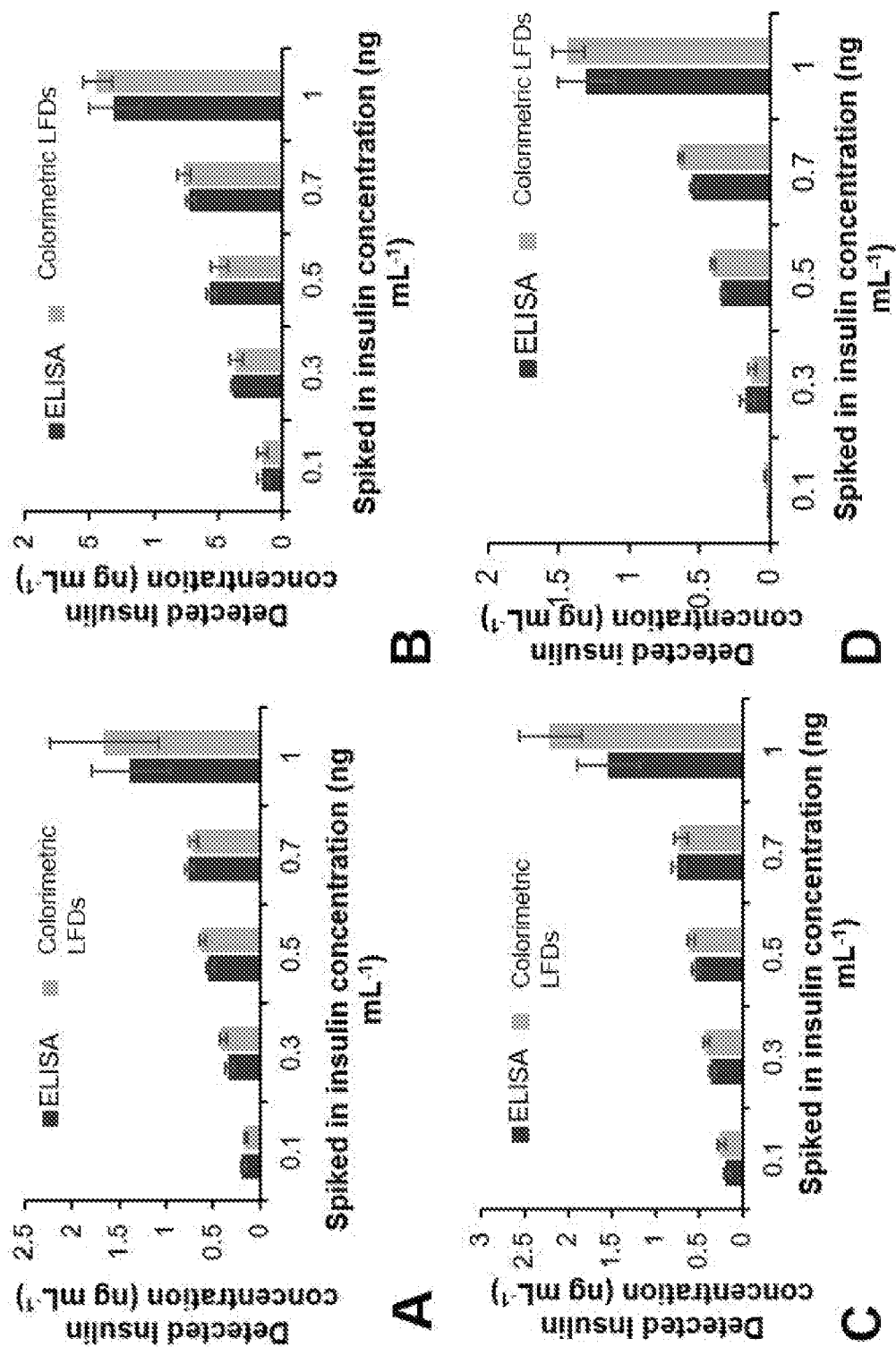
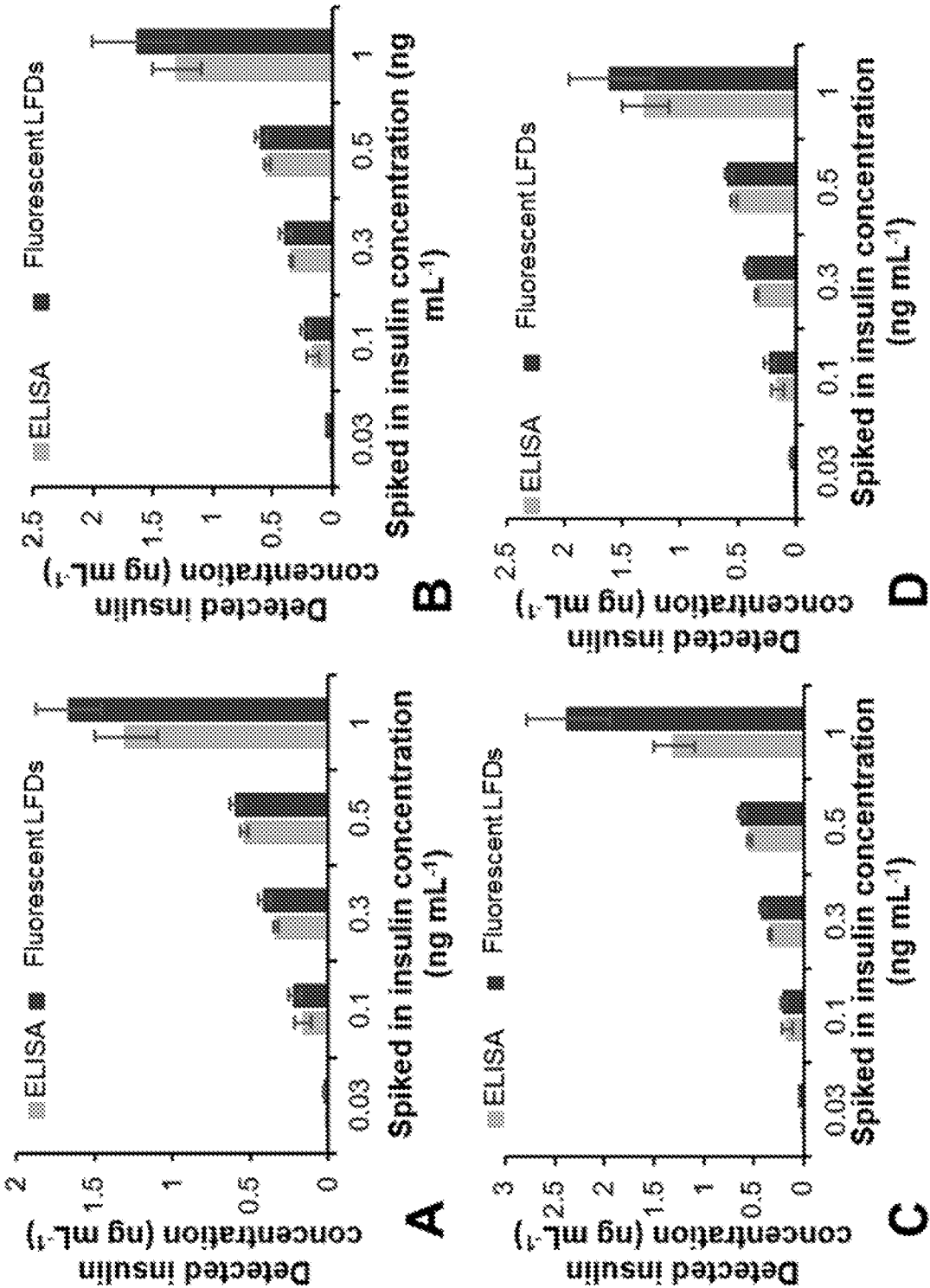
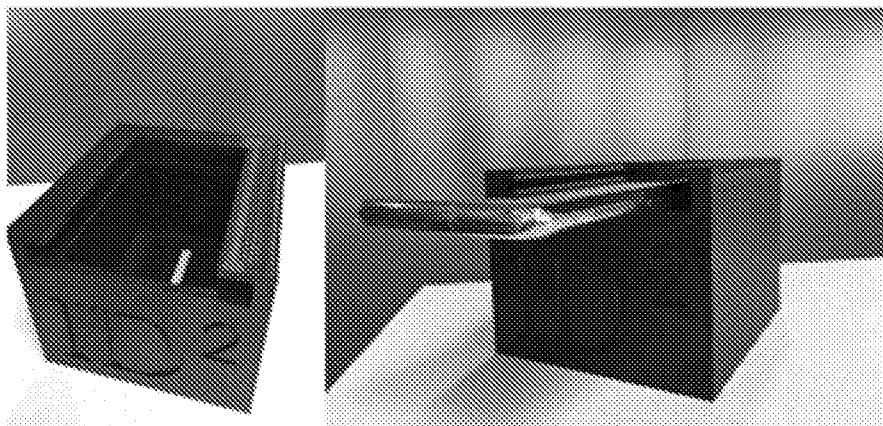
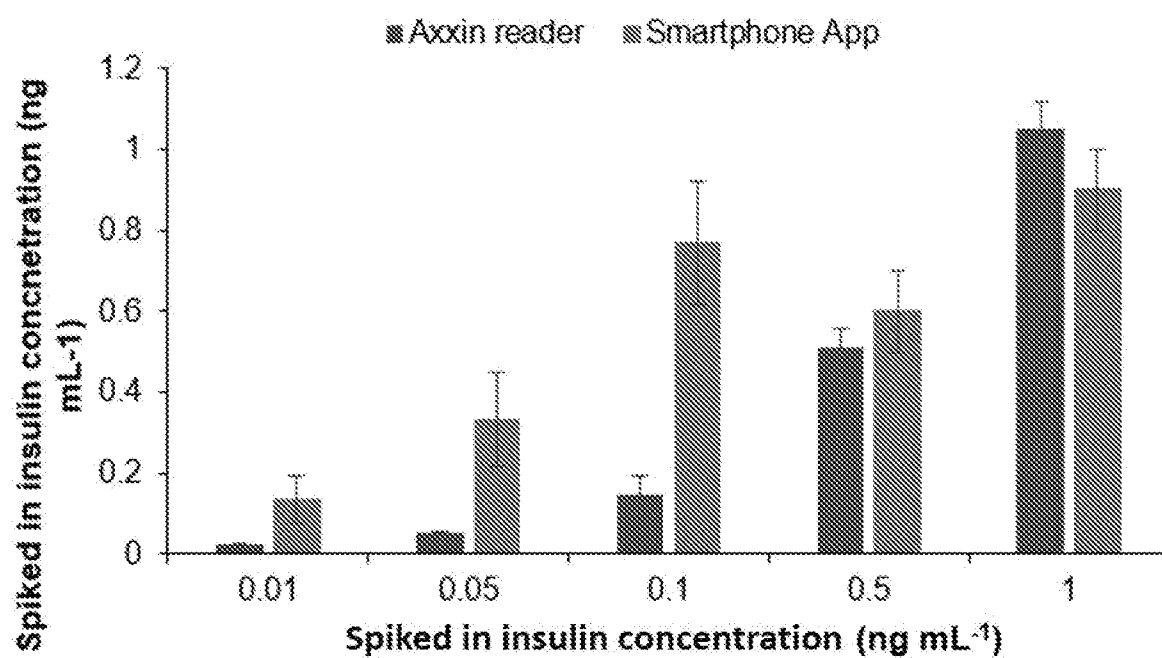


Figure 29



**Figure 30****Figure 31**

## INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/AU2019/051261**

## A. CLASSIFICATION OF SUBJECT MATTER

**G01N 21/78 (2006.01)**      **C12N 15/115 (2010.01)**      **G01N 33/74 (2006.01)**      **C12Q 1/6816 (2018.01)**  
**G01N 33/543 (2006.01)**    **G01N 33/58 (2006.01)**    **G01N 33/551 (2006.01)**

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)

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

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

Google: "lateral flow device", insulin, development manual, rolling circle amplification, "glucose encapsulated liposome", aptamer  
 Google Scholar: "lateral flow device", "rolling circle amplification", "lateral flow", "glucose encapsulated liposome", aptamer, signal amplification, DNA, RCA

PATENW: lateral flow, LFA, LFD, Dipstick, rapid test, test strip, quick test, insulin, aptamer, rolling circle amplification, graphene oxide, glucose loaded liposome, glucose encapsulated liposome, microvesicle, nanovesicle, vesicle

STN: MEDLINE, HCAPLUS, BIOSIS, EMBASE: insulin, aptamer, test strip, lateral flow, glucose loaded liposome, rolling circle amplification

AusPat: Newsouth Innovations Pty Limited and Guozhen Liu

cSpacenet: Newsouth Innovations Pty Limited and Guozhen Liu

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
|           | Documents are listed in the continuation of Box C                                  |                       |

☒ 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                                                                | "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                                              |
| "D" document cited by the applicant in the international application                                                                                                    | "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                                                                     |
| "E" earlier application or patent but published on or after the international filing date                                                                               | "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 |
| "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) | "&" document member of the same patent family                                                                                                                                                                                                    |
| "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                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search  
29 January 2020

Date of mailing of the international search report  
29 January 2020

## Name and mailing address of the ISA/AU

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 Telephone No. +61262832116

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
  - a. ☐ forming part of the international application as filed:
    - ☐ in the form of an Annex C/ST.25 text file.
    - ☐ on paper or in the form of an image file.
  - b. ☒ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
  - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
    - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
    - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

| INTERNATIONAL SEARCH REPORT                           |                                                                                                                                                                                                                                                                                                                         | International application No. |
|-------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                         | PCT/AU2019/051261             |
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                      | Relevant to claim No.         |
| Y                                                     | Chen, A. et al., 'Replacing antibodies with aptamers in lateral flow immunoassay', Biosensors and Bioelectronics. 2015, vol.71, pages 230-242<br>Abstract, title, figure 6, section 4.2, page 239, Antibodies' limitations and aptamers' opportunities in LFIA, Principles of lateral flow assays, section 1.1, table 1 | 1-27 and 72-81                |
| Y                                                     | WO 2016/168656 A1 (Lee, Ji Hoon) 20 October 2016<br>Table 1                                                                                                                                                                                                                                                             | 1-27 and 55-81                |
| Y                                                     | Cha, T. et al., 'Optical Nanosensor Architecture for Cell-Signalling Molecules Using DNA Aptamer-Coated Carbon Nanotubes', American Chemical Society. 2011, vol.5, pages 4236-4244<br>Methods: Materials                                                                                                                | 1-27 and 55-81                |
| A                                                     | Jauset-Rubio, M. et al., 'Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay', Nature Scientific Reports. 2016, vol.6, pages 1-10<br>page 2 3rd paragraph                                                                                                                      |                               |
| Y                                                     | WO 2017/138969 A1 (Washington State University) 17 August 2017<br>page 27, claim 6, and claim 1, page 2 line 24 and claim 2, page 16 line 5, page 28, page 19 line 18                                                                                                                                                   | 55-71                         |
|                                                       |                                                                                                                                                                                                                                                                                                                         |                               |

| <b>INTERNATIONAL SEARCH REPORT</b><br>Information on patent family members                                                                                                                                                                                            |                         | International application No.<br><b>PCT/AU2019/051261</b> |                            |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|-----------------------------------------------------------|----------------------------|
| This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information. |                         |                                                           |                            |
| <b>Patent Document/s Cited in Search Report</b>                                                                                                                                                                                                                       |                         | <b>Patent Family Member/s</b>                             |                            |
| <b>Publication Number</b>                                                                                                                                                                                                                                             | <b>Publication Date</b> | <b>Publication Number</b>                                 | <b>Publication Date</b>    |
| WO 2016/168656 A1                                                                                                                                                                                                                                                     | 20 October 2016         | WO 2016168656 A1<br>US 2018275120 A1                      | 20 Oct 2016<br>27 Sep 2018 |
| WO 2017/138969 A1                                                                                                                                                                                                                                                     | 17 August 2017          | WO 2017138969 A1                                          | 17 Aug 2017                |
| <b>End of Annex</b>                                                                                                                                                                                                                                                   |                         |                                                           |                            |
| <div> <div>Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.</div> <div>Form PCT/ISA/210 (Family Annex)(July 2019)</div> </div>                                                               |                         |                                                           |                            |