SYSTEMS, COMPOSITIONS, AND METHODS FOR TARGET ENTITY DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[1] This application claims the benefit of U.S. Provisional Application No. 62/812,878 filed March 01, 2019, and U.S. Provisional Application No. 62/962,722 filed January 17, 2020, the contents of each of which are hereby incorporated herein in their entirety.

BACKGROUND

[2] Early detection of cancer greatly increases the chance of successful treatment. However, many cancers still lack effective screening recommendations. Typical challenges for cancer-screening tests include limited sensitivity and specificity. A high rate of false-positive results can be of particular concern, as it can create difficult management decisions for clinicians and patients who would not want to unnecessarily administer (or receive) anti-cancer therapy that may potentially have undesirable side effects. Conversely, a high rate of false-negative results fails to satisfy the purpose of the screening test, as patients who need therapy are missed, resulting in a treatment delay and consequently a reduced possibility of success.

SUMMARY

[2.1] The present disclosure, among other things, provides insights and technologies for achieving effective cancer screening. In one aspect, the present disclosure refers to a method comprising: (a) contacting a sample that may comprise a biological entity of interest with at least one set of detection probes, each directed to a target, which set comprises at least a first detection probe for a first target and a second detection probe for a second target, so that a combination comprising the entity of interest and the set of detection probes is generated, wherein the first detection probe comprises a first target-binding moiety and a first oligonucleotide domain coupled to the first target-binding moiety, the first oligonucleotide domain comprising a first double-stranded portion and a first single-stranded overhang extended from one end of the first oligonucleotide domain; wherein the second detection probe comprises a second target-binding moiety and a second oligonucleotide domain coupled to the second target-binding moiety, the second oligonucleotide domain comprising a second double-stranded portion and a second single-stranded overhang extended from one end of the second oligonucleotide domain, wherein the second single-stranded overhang comprises a nucleotide sequence complementary to at least a portion of the first single-stranded overhang and can thereby hybridize to the first single-stranded overhang; and

wherein the first oligonucleotide domain and the second oligonucleotide domain have a combined length such that, when the first and second targets are simultaneously present on the entity of interest and the probes of the set of detection probes are bound to their respective targets on the entity of interest, the first single-stranded overhang and the second single-stranded overhang can hybridize together; (b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the entity of interest such that, when the entity of interest comprises the first target and the second target, the first detection probe and the second detection probe bind to the entity of interest to form a double-stranded complex; (c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template comprising a strand of the first double-stranded portion and a strand of the second double-stranded portion; and (d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the sample of the entity of interest comprising the first target and the second target.

[2.2] In another aspect, the present disclosure refers to a method of detecting cancer, the method comprising steps of: detecting on surfaces of intact extracellular vesicles from a human blood sample co-localization of at least two biomarkers whose combined expression level has been determined to be associated with cancer; comparing the detected co-localization level with the determined level; and detecting cancer when the detected co-localization level is at or above the determined level.

[2.3] In another aspect, the present disclosure refers to a method comprising steps of contacting a sample comprising exosomes with a set of detection probes that specifically bind to surface biomarkers on the exosomes to detect cancer-associated exosomes in the sample with a specificity within a range of 95% to 100% and sensitivity within a range of 30% to 100%.

[2.4] In another aspect, the present disclosure refers to a method comprising steps of: capturing exosomes from a biological sample with a capture agent that selectively interacts with a cancer-specific surface protein biomarker on the exosomes; and contacting the captured exosomes with at least one set of at least two detection probes that each selectively interacts with a surface protein biomarker on the exosomes; and detecting a product formed when the at least two detection probes of the set are in sufficiently close proximity, such detection indicating co-localization of the surface protein biomarkers.

[2.5] In another aspect, the present disclosure refers to a method comprising steps of: contacting a sample comprising exosomes with a set of probes that specifically bind to surface biomarkers on the exosomes to detect cancer-associated exosomes in the sample, wherein: (i) each probe in the set comprises a target binding moiety directed to a surface biomarker on the exosomes; and (ii) the set comprises at least one capture probe and at least two detection probes, wherein each detection probe further comprises a detection moiety.

[2.6] In another aspect, the present disclosure refers to a method comprising steps of: performing a proximity assay that detects a surface biomarker signature on exosomes from a human subject, the step of performing being performed a period of time after a performance of a prior assay to detect the surface biomarker signature on exosomes from the human subject; and comparing results of the performed assay with those of the prior assay.

[2.7] In another aspect, the present disclosure refers to a method comprising steps of: contacting exosomes with at least two detection probes, wherein each detection probe comprises (i) a binding moiety; and (ii) an oligonucleotide entity, wherein the binding moiety is the same and the oligonucleotide entities complement one another.

[2.8] In another aspect, the present disclosure refers to a complex comprising: (a) an exosome having at least one target biomarker on its surface; and (b) a first detection probe and a second detection probe each bound to the exosome, wherein each of the first detection probe and the second detection probe comprises: (i) a target binding moiety directed to a target biomarker expressed by the exosome; and (ii) an oligonucleotide domain coupled to the target binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain, wherein the single-stranded overhang portions of the first and second detection probes are hybridized to each other.

[2.9] In another aspect, the present disclosure refers to a composition comprising extracellular vesicles from a human blood sample bound to a set of at least two probes, each of which comprises a biomarker binding moiety and an oligonucleotide domain, wherein two or more bound probes are in proximity to one another so that their oligonucleotide domains hybridize to each other to form a ligatable hybrid.

[2.10] In another aspect, the present disclosure refers to a complex comprising: (a) an exosome comprising a cancer-associated target biomarker signature; and (b) at least a first detection probe and a second detection probe each bound to the exosome, wherein each of the detection probes comprise: (i) a target binding moiety directed; to the target biomarker signature; and (ii) an oligonucleotide domain coupled to the target binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain, wherein the single-stranded overhang portions of the detection probes are at least partially complementary.

[2.11] In yet another aspect, the present disclosure refers to a set of probes, each of which comprises: (a) a biomarker binding moiety that specifically binds to a surface biomarker on extracellular vesicles from cancer cells; and (b) an oligonucleotide domain, wherein the oligonucleotide domains of probes within the set are arranged and constructed so that, when the probes are bound to their target biomarkers, their oligonucleotide domains hybridize to one another to form a ligatable hybrid only when the target biomarkers are in proximity to one another.

[3] In some examples, provided technologies are effective for detection of early- stage cancers. In some examples, provided technologies are effective even when applied to populations comprising or consisting of asymptomatic individuals (*e.g.*, due to sufficiently high sensitivity and/or low rates of false-positive and/or false- negative results). In some examples, provided technologies are effective when applied to populations comprising or consisting of individuals (*e.g.*, asymptomatic individuals) without hereditary risk in developing cancer. In some examples, provided technologies may be or include one or more compositions (*e.g.*, molecular entities or complexes, systems, cells, collections, combinations, kits, *etc*.) and/or methods (*e.g.*, of making, using, assessing, *etc*.), as will be clear to one skilled in the art reading the disclosure provided herein.

[4] In some examples, the present disclosure identifies the source of a problem with certain prior technologies including, for example, certain conventional approaches to cancer detection and diagnosis. For example, the present disclosure appreciates that many conventional diagnostic assays, *e.g.*, based on cell-free nucleic acids, circulating tumor cells, proteins, serum proteins, and/or bulk analysis of extracellular vesicles, can be time-consuming, costly, and/or lacking sensitivity and/or specificity sufficient to provide a reliable and comprehensive diagnostic assessment. In some examples, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by developing a target entity detection approach based on interaction and/or co-localization of molecules or epitopes on individual target entities (*e.g.*, biological entities).

[5] In some examples, the present disclosure, among other things, provides insights that screening of asymptotic individuals, *e.g.*, regular screening prior to or otherwise in absence of developed symptom(s), can be beneficial, and even important for effective management (*e.g.*, successful treatment) of cancer. Alternatively or additionally, in some examples, the present disclosure further provides insights that screening (*e.g.*, regular screening) for different types of cancer (*e.g.*, for a plurality of different cancers) can be beneficial, and even important for effective management (*e.g.*, successful treatment) of cancer. In some examples, the present disclosure provides cancer screening systems that can be implemented, for example, to detect cancer, including early- stage cancer, in some examples in asymptomatic individuals (*e.g.*, without hereditary risks in cancer). In some examples, provided technologies are implemented to achieve regular screening of asymptomatic individuals (*e.g.*, without hereditary risks in cancer) and/or for multiple cancers. The present disclosure provides, for example, compositions (*e.g.*, reagents, kits, components, *etc*.), and methods of providing and/or using them, including strategies that involve regular testing of one or more individuals (*e.g.*, asymptomatic individuals). The present disclosure defines usefulness of such systems, and provides compositions and methods for implementing them.

[6] In some examples, provided technologies achieve detection (*e.g.*, early detection, *e.g.*, in asymptomatic individual(s) and/or population(s)) of one or more features (*e.g.*, incidence, progression,

responsiveness to therapy, recurrence, *etc*.) of cancer and/or a plurality of cancers, with sensitivity and/or specificity (*e.g.*, rate of false- positive and/or false- negative results) appropriate to permit useful application of provided technologies to single-time and/or regular (*e.g.*, periodic) assessment. In some examples, provided technologies are useful in conjunction with individuals' periodic physical examination. In some examples, provided technologies are useful in conjunction with treatment regimen(s); in some examples, provided technologies may improve one or more characteristics (*e.g.*, rate of success according to an accepted parameter) of such treatment regimen(s).

[7] In some examples, provided are technologies for use in detecting an entity of interest (*e.g.*, a biological or chemical entity, such as a cell, an extracellular vesicle, or an analyte, *etc*.) comprising at least two or more targets (*e.g.*, molecular targets), which in some examples such at least two or more targets may be the same, while in some examples, such at least two or more targets may be distinct. In some examples, the present disclosure provides systems comprising a plurality (*e.g.*, at least two, at least three, or more) of detection probes each for a specific such target. In some examples, a provided system may comprise two or more (*e.g.*, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) detection probes. In some examples, individual detection probes may be directed at different targets. In some examples, two or more individual detection probes may be directed at the same target. In some examples, a provided system comprises two or more different detection probes directed at different targets, and optionally may include at least one additional detection probe also directed at a target to which another detection probe is directed. In some examples, a provided system comprises a plurality of subsets of detection probes, each subset of which comprises two or more detection probes directed at the same target.

[8] Typically, a detection probe as provided and/or utilized herein comprises a target binding moiety and an oligonucleotide domain coupled to the target binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang extended from at least one end of the oligonucleotide domain. In many examples, at least a portion of a single-stranded overhang is designed such that it is complementary to a single-stranded overhang of a second detection probe, thus forming a double-stranded complex with a second detection probe through hybridization of the complementary single-stranded overhangs. In many examples, an oligonucleotide domain of a detection probe is configured to have a length such that when a first detection probe and a second detection probe bind to their respective targets on the same entity of interest (*e.g.*, on a particular biological entity of interest), the first single-stranded overhang and the second single-stranded overhang are in sufficiently close proximity to permit hybridization.

[9] In some examples involving detection probes as described and/or utilized herein, the corresponding oligonucleotide domain has a single-stranded overhang of at least 4 to 15 nucleotides in

length. In some examples, a single-stranded overhang has a nucleotide sequence preferentially selected for ligation by a specific nucleic acid ligase of interest (*e.g.*, a DNA ligase such as a T4 or T7 ligase). For example, such a single-stranded overhang may have a nucleotide sequence of GAGT.

[10] In some examples involving detection probes as described and/or utilized herein, the length of the corresponding oligonucleotide domain can vary with, *e.g.*, physical characteristics of an entity of interest (*e.g.*, a biological entity of interest), and/or selection and localization of targets (*e.g.*, molecular targets) in an entity of interest (*e.g.*, a biological entity of interest). For example, when a biological entity is or comprises an extracellular vesicle (*e.g.*, an exosome), the oligonucleotide domain of detection probes can each independently have a length, for example, of about 20 nm to about 200 nm such that their respective single-stranded overhangs are in sufficiently close proximity to anneal to each other when the corresponding detection probes are bound to the same extracellular vesicle.

[11] In some examples involving at least one set of two (*i.e.,* at least one pair of) detection probes in a system as described and/or utilized herein, oligonucleotide domains of such detection probes are configured such that when single-stranded overhangs of a first detection probe and a second detection probe anneal to each other to form a double-stranded complex, each respective target binding moiety is located at each end of the double-stranded complex. In some examples of such a double-stranded complex, both strands of the double-stranded complex are ligatable in the presence of a nucleic acid ligase, *e.g.*, for amplification and/or detection.

[12] In some examples involving at least three or more ($n \geq 3$) detection probes in a system as described and/or utilized herein, when single-stranded overhangs of detection probes anneal to each respective partner(s) to form a double-stranded complex, at least (n-2) target binding moiety/moieties is/are present at internal position(s) of the double-stranded complex. In some examples of such a double-stranded complex, it is desirable to have internal target binding moieties present in a single strand of the double-stranded complex such that another strand of the double-stranded complex is free of any internal target binding moieties and is thus ligatable in the presence of a nucleic acid ligase, *e.g.*, for amplification and/or detection.

[13] In some examples, the present disclosure utilizes a set of detection probes (*e.g.,* two or more detection probes) that together are specific for a cancer (*e.g.,* a particular cancer and/or stage of cancer as described herein), though one or more individual such probes may be directed to a target that itself is not specific to the cancer. For example, in some examples, a useful set of detection probes may comprise at least one detection probe directed to a target specific for the relevant cancer (*i.e.,* a cancer-specific target), and may further comprise at least one detection probe directed to a target that is not necessarily or completely specific for the relevant cancer (*e.g.,* that may also be found on some or all cells that are not cancerous, are not of the particular cancer, and/or are not of the particular stage of interest). That is, as

will be appreciated by those skilled in the art reading the present specification, so long as the set of detection probes utilized in accordance with the present invention is or comprises a plurality of individual detection probes that together are specific for the relevant target biological entities of interest (*e.g.*, cancer cells of interest or extracellular vesicles secreted by cancer cells) (*i.e.,* sufficiently distinguish the relevant target biological entities (*e.g.*, cancer cells of interest or extracellular vesicles secreted by cancer cells) for detection from other biological entities not of interest for detection), the set is useful in accordance with certain examples of the present disclosure.

[14] In some examples where a strand of a double-stranded complex comprises at least one or more internal target binding moieties, the strand comprises a gap between an end of an oligonucleotide strand of a detection probe to which the internal target binding moiety is coupled and an end of an oligonucleotide strand of another detection probe. In some examples, the size of a gap within a strand of a double-stranded complex is large enough such that the strand becomes non-ligatable in the presence of a nucleic acid ligase. In some examples, the gap may be at least 2 to 8 nucleotides in size. In some examples, the gap is about 6 nucleotides in size.

[15] In some examples involving detection probes as described and/or utilized herein, a target-binding moiety is or comprises an antibody agent directed at a specific target (*e.g.*, a specific molecular target). In some examples, a target-binding moiety is or comprises an aptamer for a specific target (*e.g.*, a specific molecular target). For example, in some examples, a target-binding moiety of a detection probe can be or comprise an antibody agent against a cancer-associated target such as a cancer-associated epitope. For example, in some examples, a cancer-associated target can be or comprise a target that is associated with more than one cancer (*i.e.*, at least two or more cancers). In some examples, a cancer-associated can be or comprise a generic target that is typically associated with cancers. In some examples, a cancer-associated target can be or comprise a target that is associated with cancers of a specific tissue. In some examples, a cancer-associated target can be or comprise a target that is specific to a particular cancer.

[16] In some examples involving detection probes as described and/or utilized herein, a target-binding moiety of a detection probe may be directed to a tissue-specific target (*e.g.,* present in a normal healthy tissue and/or a diseased tissue such as a tumor). In some examples, a target-binding moiety may be directed to a target that is specifically associated with a normal healthy condition of a subject.

[17] In some examples involving detection probes as described and/or utilized herein, an oligonucleotide domain is coupled to a target binding moiety by a means of covalent attachment (*e.g.*, via a bond and/or a linker). Methods to covalently couple an oligonucleotide to various agents, *e.g.*, peptides, nucleic acids, or antibodies, are known in the art and can be used to couple an oligonucleotide domain to a target binding moiety to form a detection probe as provided and/or utilized herein.

[18] In some examples, a system appropriate for use in accordance with the present disclosure can comprise a control probe (*e.g.,* in addition to target-specific detection probes). For example, in some examples, a control probe is configured to bind to a control reference such that its binding to an entity of interest (*e.g.*, a biological entity of interest) inhibits or precludes generation of a detection signal (*e.g.,* by inhibiting or precluding ligation of two or more detection probes and/or amplification of a template such as a template resulting from ligation of two or more detection probes).

[19] In some examples, technologies described herein are useful, among other things, in detecting one or more entities of interest (*e.g.,* one or more biological and/or chemical entities) in a sample (*e.g.,* in a biological, environmental, or other sample), through interaction with two or more targets (*e.g.,* molecular targets, which may, in some examples, represent different sites on a single molecular target, complex, agent, *etc*., or, more commonly, may be on different molecular targets). Those skilled in the art, reading the present disclosure will recognize that provided technologies are useful for a wide variety of applications and/or purposes. Such a skilled person will further appreciate that certain particular examples described herein relate to methods of using a plurality (*e.g.*, at least 2, at least 3, or more) of detection probes as described and/or utilized herein. In some examples, a method comprises contacting an entity of interest (*e.g.*, a biological entity) in a sample with a set of detection probes appropriate for use in accordance with the present disclosure.

[20] In some examples, a method provided herein can comprise immobilizing an entity of interest (*e.g.*, a biological entity) to be assayed on a solid substrate. An exemplary solid substrate may be or comprise a bead or a surface. In some examples, a solid substrate may be or comprise a capture surface (*e.g*., an entity capture surface) of an assay chamber, including, *e.g.*, a bead, filter, matrix, membrane, plate, tube, and/or well.

[21] In some examples, a method provided herein comprises allowing a set of detection probes (*e.g.*, as described and/or utilized herein) to bind to one or more entities of interest (*e.g.*, one or more biological entities) in a sample such that detection probes bind to respective targets (*e.g.*, molecular targets), if present, in the entities of interest (*e.g.*, biological entities) to form one or more double-stranded complexes. In some examples, a double-stranded complex is formed by direct hybridization or annealing of complementary single-stranded overhangs of detection probes bound to an entity of interest (*e.g.*, a biological entity). Therefore, in at least some examples, no connector oligonucleotides are necessary to indirectly connect oligonucleotide domains of detection probes; in some examples, such connector oligonucleotides are not utilized. Accordingly, in some examples, a method provided herein does not comprise, after an entity of interest (*e.g.*, a biological entity) is contacted with a plurality of detection probes, adding a connector oligonucleotide that is capable of binding to at least a portion of an

oligonucleotide domain of a first detection probe and at least a portion of an oligonucleotide domain of a second detection probe.

[22] A double-stranded complex (resulted from contacting an entity of interest (*e.g.*, a biological entity) with detection probes, *e.g.*, as described and/or utilized herein) can be contacted with a nucleic acid ligase to generate a ligated template comprising at least an oligonucleotide strand of a first detection probe and an oligonucleotide strand of a second detection probe. Detection of such a ligated template provides information as to whether an entity of interest (*e.g.*, a biological entity) in a sample is positive or negative for targets to which detection probes are directed. For example, a detectable level (*e.g.*, a level that is above a reference level, *e.g.*, by at least 10% or more, where in some examples, a reference level may be a level observed in a negative control sample, such as a sample in which an entity of interest comprising such targets is absent) of such a ligated template is indicative of a tested entity of interest (*e.g.*, a biological entity) comprising targets (*e.g.*, molecular targets) of interest, whereas a non-detectable level (*e.g.*, a level that is below the threshold of a detectable level) of such a ligated template indicates that at least one of targets (*e.g.*, molecular targets) of interest is absent from a tested entity of interest (*e.g.*, a biological entity).

[23] A ligated template can be detected by an appropriate nucleic acid detection method known in the art. For example, in some examples, a ligated template is detected by performing amplification of the ligated template, optionally followed by detection of the presence of the amplification product. An exemplary nucleic acid detection method involves quantitative polymerase chain reaction.

[24] Technologies provided herein can be applied to a sample of interest comprising an entity of interest (*e.g.*, a biological or chemical entity, such as an extracellular vesicle and/or an analyte) to be assayed. For example, in some examples, a sample may be or comprise a biological sample. In some examples, a sample may be or comprise an environmental sample. In some examples, a sample may be or comprise a primary sample. In some examples, a sample may be or comprise a processed sample. For example, in some examples, a sample may be processed to isolate one or more entities of interest to be assayed.

[25] In some examples where a sample comprises or is a biological sample, such a sample can be derived from a plasma or blood sample of a subject (*e.g.*, a human subject) in need of such an assay. In some examples, a biological sample can be or comprise a primary sample (*e.g.*, a tissue or tumor sample) from a subject (*e.g.*, a human subject) in need of such an assay. In some examples, a biological sample can be processed to separate one or more entities of interest from non-target entities of interest, and/or to enrich one or more entities of interest. In some examples, an entity of interest present in a sample may be or comprise a biological entity, *e.g.*, a cell or an extracellular vesicle (*e.g.*, an exosome). In some examples, such a biological entity may be processed or contacted with a chemical reagent, *e.g.*, to

stabilize and/or crosslink targets to be assayed in the biological entity and/or to reduce non-specific binding with detection probes. In some examples, a biological entity is or comprises a cell, which may be optionally processed, *e.g.*, with a chemical reagent for stabilizing and/or crosslinking targets (*e.g.*, molecular targets) and/or for reducing non-specific binding. In some examples, a biological entity is or comprises an extracellular vesicle (*e.g.*, an exosome), which may be optionally processed, *e.g.*, with a chemical reagent for stabilizing and/or crosslinking targets (*e.g.*, molecular targets) and/or for reducing non-specific binding.

[26] In some examples, the present disclosure, among other things, provide technologies (including systems, compositions, and methods) that solve problems associated with conventional cancer diagnostics, *e.g.*, based on cell-free nucleic acids, serum proteins, and/or bulk analysis of extracellular vesicles, by detecting co-localization of a target biomarker signature of cancer in individual extracellular vesicles, which comprises at least one extracellular vesicle-associated membrane-bound polypeptide and at least one target biomarker selected from the group consisting of surface protein biomarkers, internal protein biomarkers, and RNA biomarkers. In some examples, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by detecting such target biomarker signature of cancer using a target entity detection approach (*e.g.*, ones described herein) which is based on interaction and/or co-localization of at least two or more target entities (*e.g.*, a target biomarker signature) in individual extracellular vesicles.

[27] In some examples, provided are technologies for use in classifying a subject (*e.g.*, an asymptomatic subject) as having or being susceptible to cancer. In some examples, the present disclosure provides methods or assays for classifying a subject (*e.g.*, an asymptomatic subject) as having or being susceptible to cancer. In some examples, a provided method or assay comprises (a) detecting, in a blood-derived sample from a subject in need thereof, extracellular vesicles expressing a target biomarker signature of cancer, the target biomarker signature comprising: at least one extracellular vesicle-associated membrane-bound polypeptide and at least one target biomarker selected from the group consisting of: surface protein biomarkers, intravesicular protein biomarkers, and intravesicular RNA biomarkers; (b) comparing sample information indicative of level of the target biomarker signature-expressing extracellular vesicles in the blood-derived sample to reference information including a reference threshold level; and (c) classifying the subject as having or being susceptible to cancer when the blood-derived sample shows an elevated level of target biomarker signature-expressing extracellular vesicles relative to the reference threshold level.

[28] In some examples, an extracellular vesicle-associated membrane-bound polypeptide for use in a target biomarker signature of cancer used and/or described herein may be or comprise a tumor-

specific biomarker and/or a tissue-specific biomarker. In some examples, such an extracellular vesicle-associated membrane-bound polypeptide may be or comprise a non-specific marker, *e.g.*, it is present in one or more non-target tumors, and/or in one or more non-target tissues.

[29] In some examples, a target biomarker signature of cancer may comprise an extracellular vesicle-associated membrane-bound polypeptide and at least one additional target surface protein biomarker.

[30] In some examples, a target biomarker signature of cancer may comprise an extracellular vesicle-associated membrane-bound polypeptide and at least one target intravesicular RNA (*e.g.*, mRNA) biomarker.

[31] In some examples, a target biomarker signature of cancer may comprise an extracellular vesicle-associated membrane-bound polypeptide (*e.g.*, ones described herein) and at least one additional target intravesicular protein biomarker.

[32] In some examples, a reference threshold level for use in a provided method or assay described herein is determined by levels of target biomarker signature-expressing extracellular vesicles observed in comparable samples from a population of non-cancer subjects.

[33] In some examples, an extracellular vesicle-associated membrane-bound polypeptide included in a target biomarker signature may be detected using antibody-based agents. In some examples, such an extracellular vesicle-associated membrane-bound polypeptide may be detected using a capture assay comprising an antibody-based agent. For example, in some examples, a capture assay for detecting the presence of an extracellular vesicle-associated membrane-bound polypeptide in an extracellular vesicle may involve contacting a blood-derived sample comprising extracellular vesicles with a capture agent directed to such an extracellular vesicle-associated membrane-bound polypeptide. In some examples, such a capture agent may comprise a binding moiety directed to an extracellular vesicle-associated membrane-bound polypeptide (*e.g.*, ones described herein), which may be optionally conjugated to a solid substrate. Without limitations, an exemplary capture agent for an extracellular vesicle-associated membrane-bound polypeptide may be or comprise a solid substrate (*e.g.*, a magnetic bead) and a binding moiety (*e.g.*, an antibody agent) directed to an extracellular vesicle-associated membrane-bound polypeptide.

[34] In some examples, a target biomarker included in a target biomarker signature may be detected using appropriate methods known in the art, which may vary with types of analytes to be detected (*e.g.*, surface proteins, intravesicular proteins, intravesicular RNA (*e.g.*, mRNA)). For example, a person skilled in the art, reading the present disclosure, will appreciate that a surface

protein biomarker and/or an intravesicular protein biomarker may be detected using antibody-based agents in some examples, while in some examples, an intravesicular RNA (*e.g.*, mRNA) biomarker may be detected using nucleic acid-based agents, *e.g.*, using quantitative reverse transcription PCR.

[35] For example, in some examples where a target biomarker is or comprises a surface protein biomarker and/or an intravesicular protein marker, such a target biomarker may be detected involving a proximity ligation assay, *e.g.*, following a capture assay (*e.g.*, ones as described herein) to capture extracellular vesicles that express an extracellular vesicle-associated membrane-bound polypeptide (*e.g.*, ones as used and/or described herein). In some examples, such a proximity ligation assay may comprise contacting a blood-derived sample comprising extracellular vesicles with a set of detection probes, each directed to a target biomarker, which set comprises at least two distinct detection probes, so that a combination comprising the extracellular vesicles and the set of detection probes is generated, wherein the two detection probes each comprise: (i) a binding moiety directed to a surface protein biomarker and/or an intravesicular protein biomarker; and (ii) an oligonucleotide domain coupled to the binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain. Such single-stranded overhang portions of the detection probes are characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle. Such a combination comprising the extracellular vesicles and the set of detection probes is then maintained under conditions that permit binding of the set of detection probes to their respective targets on the extracellular vesicles such that the detection probes can bind to the same extracellular vesicle to form a double-stranded complex. Such a double-stranded complex can be detected by contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template; and detecting the ligated template. The presence of such a ligated template is indicative of presence of extracellular vesicles that are positive for a target biomarker signature of cancer. While such a proximity ligation assay may perform better, *e.g.*, with higher specificity and/or sensitivity than other existing proximity ligation assays, a person skilled in the art reading the present disclosure will appreciate that other forms of proximity ligation assays that are known in the art may be used instead.

[36] In some examples where a target biomarker is or comprises an intravesicular RNA (*e.g.*, mRNA) marker, such a target biomarker may be detected involving a nucleic acid detection assay. In some examples, an exemplary nucleic acid detection assay may be or comprise reverse-transcription PCR.

[37] In some examples where a target biomarker is or comprises an intravesicular biomarker (*e.g.*, an intravesicular protein biomarker and/or an intravesicular RNA (*e.g.*, mRNA) biomarker), such a target biomarker may be detected involving, prior to a detection assay (*e.g.*, a proximity ligation assay as described herein), a sample treatment (*e.g.*, fixation and/or permeabilization) to expose intravesicular biomarker(s) for subsequent detection.

[38] The present disclosure, among other things, recognizes that detection of a single cancer-associated serum protein or biomarker or a plurality of cancer-associated biomarkers based on a bulk sample (*e.g.*, a bulk sample of extracellular vesicles), rather than at a resolution of a single extracellular vesicle, typically does not provide sufficient specificity and/or sensitivity in determination of whether a subject from whom the sample is obtained is likely to be suffering from or susceptible to cancer. The present disclosure, among other things, provides technologies, including systems, compositions, and/or methods, that solve such problems, including, for example, by specifically requiring that individual target entities (*e.g.*, individual biological entities such as individual extracellular vesicles) for detection be characterized by presence of a combination of targets (*e.g.*, molecular targets). In some examples, such a combination of targets may be a target biomarker signature comprising a combination of at least one or more extracellular vesicle-associated membrane-bound polypeptides and at least one or more target biomarkers. In particular examples, the present disclosure teaches technologies that require such individual entities (*e.g.*, biological entities such as extracellular vesicles) be characterized by presence (*e.g.,* by expression) of a combination of targets (*e.g.*, molecular targets) that is cancer specific (*i.e.*, "target biomarker signature" of the relevant cancer), while biological entities (*e.g.*, extracellular vesicles) that do not comprise the targeted combination (*e.g.*, target biomarker signature) do not produce a detectable signal (*e.g.*, a level that is above a reference level, *e.g.*, by at least 10% or more, where in some examples, a reference level may be a level observed in a negative control sample, such as a sample in which individual biological entities (*e.g.*, individual extracellular vesicles) comprising such a targeted combination (*e.g.*, a target biomarker signature) are absent).

[39] Accordingly, in some examples, technologies provided herein can be useful for detection of incidence or recurrence of cancer in a subject and/or across a population of subjects. In some examples, a combination of targets (*e.g.*, a target biomarker signature) may be selected for cancer detection. In some examples, a combination of targets (*e.g.*, a target biomarker signature) may be selected for detection of a specific cancer or its stage and/or subtype thereof. In some examples, a combination of biomarkers (*e.g.*, a target biomarker signature) may be detected by a plurality of (*e.g.*,

at least two or more) pairwise or orthogonal combinations of detection probes, wherein each pair of detection probes may be directed to at least one distinct target. In some examples, a combination of biomarkers (*e.g.*, a target biomarker signature) may be detected by a set of detection probes that each are designed to hybridize to one another to form a linear complex. In some examples, a plurality of (*e.g.*, at least two or more) combinations of biomarkers may be selected for cancer detection. In some examples, a plurality of (*e.g.*, at least two or more) combinations of biomarkers may be selected for detection of a specific cancer or its stage and/or subtype thereof. In some examples, a plurality of (*e.g.*, at least two or more) orthogonal combinations of biomarkers may be selected for cancer detection. In some examples, a plurality of (*e.g.*, at least two or more) orthogonal combinations of biomarkers may be selected for detection of a specific cancer or its stage and/or subtype thereof. In some examples, a plurality of (*e.g.*, at least two or more) target biomarker signatures may be selected for cancer detection. In some examples, a plurality of (*e.g.*, at least two or more) target biomarker signatures may be selected for detection of a specific cancer or its stage and/or subtype thereof. In some examples, technologies provided herein can be used periodically (*e.g.*, every year) to screen a human subject or across a population of human subjects for early- stage cancer or cancer recurrence.

[40] In some examples, a combination (*e.g.*, a set) of detection probes may be selected for a system or a method (*e.g.*, ones described herein) for detection of a specific cancer. In some examples, a combination (*e.g.,* a set) of detection probes is selected for a system or a method (*e.g.*, ones described herein) for detection of one or more cancers. In some examples, a combination (*e.g.*, a set) of detection probes may include a plurality of (*e.g.*, at least two or more) pairwise or orthogonal combinations of detection probes, wherein each pair of detection probes may be directed to at least one distinct target. In some examples, detection probes in a combination (*e.g.*, a set) may be each designed to hybridize to one another to form a linear complex. In some examples, examples of cancers that can be detected by technologies provided herein include, but are not limited to acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer.

[41] In some examples, a subject that is amenable to technologies provided herein for detection of incidence or recurrence of cancer may be an asymptomatic human subject and/or across an asymptomatic population. Such an asymptomatic subject may be a subject who has a family history

of cancer, who has been previously treated for cancer, who is at risk of cancer recurrence after cancer treatment, who is in remission after cancer treatment, and/or who has been previously or periodically screened for the presence of at least one cancer biomarker. Alternatively, in some examples, an asymptomatic subject may be a subject who has not been previously screened for cancer, who has not been diagnosed for cancer, and/or who has not previously received cancer therapy.

[42] In some examples, a subject or population of subjects may be selected based on one or more characteristics such as age, race, genetic history, medical history, personal history (*e.g.*, smoking, alcohol, drugs, carcinogenic agents, diet, obesity, physical activity, sun exposure, radiation exposure, exposure to infectious agents such as viruses, and/or occupational hazard).

[43] In some examples, technologies provided herein can be useful for selecting therapy for a subject who is suffering from or susceptible to cancer. In some examples, a cancer therapy and/or an adjunct therapy can be selected in light of findings based on technologies provided herein.

[44] In some examples, technologies provided herein can be useful for monitoring and/or evaluating efficacy of therapy administered to a subject (*e.g.*, a cancer subject).

[45] In some examples, the present disclosure provides technologies for managing patient care, *e.g.,* for one or more individual subjects and/or across a population of subjects. To give but a few examples, in some examples, the present disclosure provides technologies that may be utilized in screening (*e.g.,* temporally or incidentally motivated screening and/or non-temporally or incidentally motivated screening, *e.g.,* periodic screening such as annual, semi-annual, bi-annual, or with some other frequency). For example, in some examples, provided technologies for use in temporally motivated screening can be useful for screening one or more individual subjects or across a population of subjects (*e.g.*, asymptomatic subjects) who are of a certain age or age group, *e.g.*, older than a certain age (*e.g.*, over 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or older). In some examples, provided technologies for use in incidentally motivated screening can be useful for screening individual subjects who may have experienced an incident or event that motivates screening for cancer as described herein. For example, in some examples, an incidental motivation relating to determination of one or more indicators of cancer or susceptibility thereto may be or comprise , *e.g.*, an incident based on their family history (*e.g.*, a close relative such as blood-related relative was previously diagnosed for cancer), identification of one or more risk factors for cancer (*e.g.*, smoking, alcohol, diet, obesity, occupational hazard, *etc*.) and/or prior incidental findings from genetic tests (*e.g.*, genome sequencing), and/or imaging diagnostic tests (*e.g.*, ultrasound, computerized tomography (CT) and/or magnetic resonance imaging (MRI) scans), development of one or more

signs or symptoms characteristic of cancer (*e.g.,* a persistent cough potentially indicative of lung cancer; a lump in breast tissue potentially indicative of breast cancer; gastrointestinal (GI) tract bleeding potentially indicative of GI cancer, or abnormal bleeding during a woman's period potentially indicative of ovarian cancer, *etc*.).

[46] In some examples, provided technologies for managing patient care can inform treatment and/or payment (*e.g.,* reimbursement for treatment) decisions and/or actions. For example, in some examples, provided technologies can provide determination of whether individual subjects have one or more indicators of incidence or recurrence of a disease or disorder (*e.g.*, cancer), thereby informing physicians and/or patients when to initiate therapy in light of such findings. Additionally or alternatively, in some examples, provided technologies can inform physicians and/or patients of treatment selection, *e.g.*, based on findings of specific responsiveness biomarkers (*e.g.*, cancer responsiveness biomarkers). In some examples, provided technologies can provide determination of whether individual subjects are responsive to current treatment, *e.g.*, based on findings of changes in one or more levels of molecular targets associated with a disease, disorder, or condition (*e.g.*, cancer), thereby informing physicians and/or patients of efficacy of such therapy and/or decisions to maintain or alter therapy in light of such findings.

[47] In some examples, provided technologies can inform decision making relating to whether health insurance providers reimburse (or not), *e.g.*, for (1) screening itself (*e.g.*, reimbursement available only for periodic/regular screening or available only for temporally and/or incidentally motivated screening); and/or for (2) initiating, maintaining, and/or altering therapy in light of findings by provided technologies. For example, in some examples, the present disclosure provides methods relating to (a) receiving results of a screening as described herein and also receiving a request for reimbursement of the screening and/or of a particular therapeutic regimen; (b) approving reimbursement of the screening if it was performed on a subject according to an appropriate schedule or response to a relevant incident and/or approving reimbursement of the therapeutic regimen if it represents appropriate treatment in light of the received screening results; and, optionally (c) implementing the reimbursement or providing notification that reimbursement is refused. In some examples, a therapeutic regimen is appropriate in light of received screening results if the received screening results detect a biomarker that represents an approved biomarker for the relevant therapeutic regimen (*e.g.,* as may be noted in a prescribing information label and/or via an approved companion diagnostic). Alternatively or additionally, the present disclosure contemplates reporting systems (*e.g.,* implemented via appropriate electronic device(s) and/or communications system(s))

that permit or facilitate reporting and/or processing of screening results, and/or of reimbursement decisions as described herein.

[48] Some examples provided herein relate to systems and kits for use in provided technologies. In some examples, a system or kit comprises at least one set of detection probes (*e.g.*, as described and/or utilized herein), each directed to a target. In some examples, a set of detection probes in a provided system or kit comprises two or more (*e.g.*, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) detection probes. In some examples, individual detection probes may be directed at different targets. In some examples, two or more individual detection probes may be directed to the same target. In some examples, a provided system or kit comprises two or more different detection probes directed at different targets, and optionally may include at least one additional detection probe also directed at a target to which another detection probe is directed.

[49] Some examples provided herein relate to systems and kits for use in provided technologies relating to biomarker detection and/or characterization. In some examples, a system or kit may comprise detection agents for a target biomarker signature of a disease or disorder (*e.g.*, cancer). In some examples, such a system or kit may comprise a capture agent for an extracellular vesicle-associated membrane-bound polypeptide present in extracellular vesicles associated with a disease or disorder (*e.g.*, cancer); and (b) at least one or more detection agents directed to one or more target biomarkers of a target biomarker signature of such a disease or disorder (*e.g.*, cancer), which may be or comprise additional surface protein biomarker(s), intravesicular protein biomarker(s), and/or intravesicular RNA (*e.g.*, mRNA) biomarker(s).

[50] In some examples, a capture agent included in a system and/or kit may comprise a binding moiety directed to an extracellular vesicle-associated membrane-bound polypeptide. In some examples, such a binding moiety may be conjugated to a solid substrate, which in some examples may be or comprise a solid substrate. In some examples, such a solid substrate may be or comprise a magnetic bead. In some examples, an exemplary capture agent included in a provided system and/or kit may be or comprise a solid substrate (*e.g.*, a magnetic bead) and an antibody agent directed to an extracellular vesicle-associated membrane-bound polypeptide conjugated thereto.

[51] In some examples where a target biomarker includes a surface protein biomarker and/or an intravesicular protein biomarker, a system and/or kit may include detection agents for performing a proximity ligation assay (*e.g.*, ones as described herein). In some examples, such detection agents for performing a proximity ligation assay may comprise a set of detection probes, each directed to a target biomarker of a target biomarker signature, which set comprises at least two detection probes,

wherein the two detection probes each comprise: (i) a polypeptide-binding moiety directed to a target biomarker; and (ii) an oligonucleotide domain coupled to the binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain, wherein the single-stranded overhang portions of the detection probes are characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle.

[52] In some examples, a provided system and/or kit may comprise a plurality (*e.g.*, 2, 3, 4, 5, or more) of sets of detection probes, each set of which comprises two or more (*e.g.*, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) detection probes. In some examples, each set may be directed to detection for a different disease, disorder, or condition. In some examples, two or more sets may be directed to detection for the same disease, disorder, or condition. In some examples, at least one or more sets (*e.g.*, at least one, at least two, at least three, at least four, at least five, or more) of detection probes may be directed to detection for cancer. For example, in some examples, each set may be directed to detection for a different cancer (*e.g.*, different cancer types associated with the same or different tissues). In some examples, two or more sets may be directed to detection of the same cancer. In some examples, a provided system and/kit may comprise at least one set of detection probes for detection of one cancer and at least one set of detection probes for detection of a different cancer (*e.g.*, pancreatic cancer). In some examples, two or more detection probes may be directed to different subtypes and/or categories of cancer. In some examples, two or more sets may be directed to detection of cancer of different stages. In some examples, two or more sets may be directed to detection of cancer of the same stage.

[53] In some examples, detection probes in a provided kit may be provided as a single mixture in a container. In some examples, multiple sets of detection probes may be provided as individual mixtures in separate containers. In some examples, each detection probe is provided individually in a separate container.

[54] In some examples where a target biomarker includes an intravesicular RNA (*e.g.*, mRNA) biomarker, such a system and/or kit may include detection agents for performing a nucleic acid detection assay. In some examples, such a system and/or kit may include detection agents for performing a quantitative reverse-transcription PCR, for example, which may comprise primers directed to intravesicular RNA (*e.g.*, mRNA) target(s).

[55] In some examples, a provided system and/or kit may comprise at least one chemical reagent, *e.g.*, to process a sample and/or entities of interest (*e.g.*, extracellular vesicles) therein. In some

examples, a provided system and/or kit may comprise at least one chemical reagent to process entities of interest (*e.g.*, biological entities of interest such as extracellular vesicles) in a sample, including, *e.g.*, but not limited to a fixation agent, a permeabilization agent, and/or a blocking agent. In some examples, a provided system and/or kit may comprise a nucleic acid ligase and/or a nucleic acid polymerase. In some examples, a provided system and/or kit may comprise one or more primers and/or probes. In some examples, a provided system and/or kit may comprise one or more pairs of primers, for example for PCR, *e.g.*, quantitative PCR (qPCR) reactions. In some examples, a provided system and/or kit may comprise one or more probes such as, for example, hydrolysis probes which may in some examples be designed to increase the specificity of qPCR (*e.g.*, TaqMan probes). In some examples, a provided system and/or kit may comprise one or more multiplexing probes, for example as may be useful when simultaneous or parallel qPCR reactions are employed (*e.g.*, to facilitate or improve readout).

[56] In some examples, a provided system and/or kit can be used for screening (*e.g.*, regular screening) and/or other assessment of individuals (*e.g.*, asymptomatic or symptomatic subjects) for detection (*e.g.*, early detection) of a disease or disorder (*e.g.*, cancer). In some examples, a provided system and/or kit can be used for screening and/or other assessment of individuals susceptible to a disease or disorder (*e.g.*, cancer) (*e.g.*, individuals with a known genetic, environmental, or experiential risk, *etc.*). In some examples, provided system and/or kits can be used for monitoring recurrence of a disease or disorder (*e.g.*, cancer) in a subject who has been previously treated. In some examples, provided systems and/or kits can be used as a companion diagnostic in combination with a therapy for a subject who is suffering from a disease or disorder (*e.g.*, cancer). In some examples, provided systems and/or kits can be used for monitoring or evaluating efficacy of a therapy administered to a subject who is suffering from a disease or disorder (*e.g.*, cancer). In some examples, provided systems and/or kits can be used for selecting a therapy for a subject who is suffering from a disease or disorder (*e.g.*, cancer). In some examples, provided systems and/or kits can be used for making a therapy decision and/or selecting a therapy for a subject with one or more symptoms (*e.g.*, non-specific symptoms) associated with a disease or disorder (*e.g.*, cancer).

[57] In some examples, a set of detection probes provided and/or utilized in a kit or system may be selected for screening (*e.g.*, regular screening) or diagnosis of a specific cancer (including, *e.g.*, but not limited to acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin

lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer).

[58] In some examples, a provided kit or system may comprise a plurality of sets of detection probes, each set comprising at least two or more detection probes for detection of a different cancer. For example, such a kit can be used to screen (*e.g.*, regularly screen) a subject or across a population of subjects for various cancers (including, *e.g.*, but not limited to acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer) in a single assay.

[59] Double-stranded complexes formed by at least two or more detection probes (*e.g.*, as described and/or utilized herein) bound to an entity of interest (*e.g.*, a chemical or biological entity, such as an extracellular vesicle or an analyte) is also within the scope of disclosure.

[60] Complexes formed by performing methods described herein and/or using systems and/or kits described herein are also within the scope of disclosure. For example, in some examples, a complex comprising: (a) an extracellular vesicle expressing a target biomarker signature, at least two of which include at least one extracellular vesicle-associated membrane-bound polypeptide and at least one target biomarker selected from the group consisting of: surface protein biomarkers, intravesicular protein biomarkers, and intravesicular RNA biomarkers, wherein the extracellular vesicle is immobilized onto a solid substrate comprising a binding moiety directed to such a extracellular vesicle-associated membrane-bound polypeptide. Such a complex further comprises at least two detection probes directed to at least one target biomarker of the target biomarker signature present in the extracellular vesicle, wherein each detection probe is bound to such a target biomarker and each comprises: (i) a binding directed to the target biomarker; and (ii) an oligonucleotide domain coupled to the binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain, wherein the single-stranded overhang portions of the detection probes are hybridized to each other.

[61] These, and other aspects encompassed by the present disclosure, are described in more detail below and in the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[62] ***Figure 1*** is a schematic diagram illustrating an exemplary workflow of profiling individual extracellular vesicles (EVs). The figure shows purification of EVs from plasma using size exclusion chromatography (SEC) and immunoaffinity capture of EVs displaying a specific membrane-bound protein marker (***Panel A***); detection of co-localized target markers (*e.g.*, intravesicular proteins or surface proteins) on captured EVs using a target entity detection assay according to some embodiments described herein (***Panel B***).

[63] ***Figures 2A-2B*** are schematic diagrams illustrating a target entity detection assay according to some embodiments described herein. ***Figure 2A*** shows, in some embodiments, a target entity detection assay using a combination of detection probes, which combination is specific for detection of cancer. In some embodiments, a duplex system including a first detection probe for a target protein 1 (*e.g.*, cancer marker 1) and a second detection probe for a target protein 2 (*e.g.*, cancer marker 2) are added to a sample comprising a biological entity (*e.g.*, extracellular vesicle or analyte). In some embodiments, detection probes each comprise a target binding moiety (*e.g.*, an antibody agent against a target protein) coupled to an oligonucleotide domain, which comprises a double-stranded portion and a single-stranded overhang extended from one end of the oligonucleotide domain. A detection signal is generated when distinct target binding moieties (*e.g.*, antibody agents against target protein 1 and target protein 2, respectively) of the first and second detection probes are localized to the same biological entity (*e.g.*, an extracellular vesicle or analyte) in close proximity such that the corresponding single-stranded overhangs hybridize to each other, thus allowing ligation of their oligonucleotide domains to occur. For example, a control entity (*e.g.*, a biological entity from a healthy subject sample) does not express one or both of target protein 1 (*e.g.*, cancer marker 1) and target protein 2 (*e.g.*, cancer marker 2), so no detection of signal can be generated. However, when a biological entity from a cancer sample expresses target protein 1 and target protein 2, and the target proteins are present within a short enough distance of each other in the same biological entity (*e.g.*, extracellular vesicle), a detection signal is generated. ***Figure 2B*** shows a non-limiting example of a double-stranded complex comprising a first detection probe and a second detection probe

CLAIMS

What is claimed is:

1. A method comprising:

(a) contacting a sample that may comprise a biological entity of interest with at least one set of detection probes, each directed to a target, which set comprises at least a first detection probe for a first target and a second detection probe for a second target, so that a combination comprising the entity of interest and the set of detection probes is generated,

wherein the first detection probe comprises a first target-binding moiety and a first oligonucleotide domain coupled to the first target-binding moiety, the first oligonucleotide domain comprising a first double-stranded portion and a first single-stranded overhang extended from one end of the first oligonucleotide domain;

wherein the second detection probe comprises a second target-binding moiety and a second oligonucleotide domain coupled to the second target-binding moiety, the second oligonucleotide domain comprising a second double-stranded portion and a second single-stranded overhang extended from one end of the second oligonucleotide domain, wherein the second single-stranded overhang comprises a nucleotide sequence complementary to at least a portion of the first single-stranded overhang and can thereby hybridize to the first single-stranded overhang; and

wherein the first oligonucleotide domain and the second oligonucleotide domain have a combined length such that, when the first and second targets are simultaneously present on the entity of interest and the probes of the set of detection probes are bound to their respective targets on the entity of interest, the first single-stranded overhang and the second single-stranded overhang can hybridize together;

(b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the entity of interest such that, when the entity of interest comprises the first target and the second target, the first

detection probe and the second detection probe bind to the entity of interest to form a double-stranded complex;

(c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template comprising a strand of the first double-stranded portion and a strand of the second double-stranded portion; and

(d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the sample of the entity of interest comprising the first target and the second target.

2. The method of claim 1, wherein the detecting comprises performing amplification of the ligated template and detecting the presence of the amplification product, optionally wherein the amplification is or comprises quantitative polymerase chain reaction.
3. The method of claim 1 or 2, wherein the entity of interest is immobilized on a solid substrate, optionally wherein the solid substrate is or comprises a bead.
4. The method of any one of claims 1-3, wherein method does not comprise, prior to the contacting of step (c), contacting the double-stranded complex with a connector oligonucleotide that associates the first oligonucleotide with the second oligonucleotide domain.
5. The method of claim 4, wherein the connector oligonucleotide hybridizes to at least a portion of the first oligonucleotide domain and at least a portion of the second oligonucleotide domain.
6. The method of any one of claims 1-5, wherein the first target-binding moiety and/or the second target-binding moiety comprise(s) an antibody agent.
7. The method of any one of claims 1-6, wherein the set of detection probes further comprises an additional detection probe for a third target, the additional detection probe comprising a third target-binding moiety and a third oligonucleotide domain coupled to the third target-binding moiety, the third oligonucleotide domain comprising a double-stranded portion and a third single-stranded overhang extended from each end of the third oligonucleotide domain.
8. The method of any one of claims 1-7, wherein the set of detection probes comprises 2-20 detection probes each for a specific target, each of the detection probes comprising a target-binding moiety and an oligonucleotide domain coupled to the target-binding

moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang extended from at least one end of the oligonucleotide domain.

9. The method of any one of claims 1-8, wherein the set of detection probes further comprises a control probe, wherein the control probe is characterized in that binding of the control probe to the entity of interest inhibits generation of a ligated template and/or inhibits amplification of a ligated template from a non-target biological entity, optionally wherein the control probe is configured to bind to a control reference.
10. The method of claim 1, wherein the biological entity of interest is or comprises a population of extracellular vesicles, optionally wherein at least one of the extracellular vesicles expreses a target biomarker signature for a disease, disorder, or condition, for example, wherein the disease, disorder, or condition is cancer, optionally wherein the cancer is selected from the group consisting of bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer.
11. The method of claim 1, wherein the sample is or comprises a blood-derived sample from a subject.
12. The method of claim 11, wherein the subject has at least one or more of the following characteristics:

a. (i) an asymptomatic subject who is susceptible to cancer;

(ii) a subject with a family history of cancer;

(iii) a subject determined to have one or more germline mutations in one or more cancer-associated genes;

(iv) an elderly subject;

(v) a subject with one or more non-specific symptoms of cancer;

(vi) a subject recommended for periodic cancer screening;

(vii) a subject diagnosed with an imaging-confirmed mass;

(viii) a subject at hereditary risk for cancer before undergoing a risk-reducing surgical intervention;

(ix) a subject with a benign tumor; and

(x) a subject who has been previously treated for cancer.

b. the sample has been subjected to size exclusion chromatography to isolate nanoparticles having a size range of interest that includes extracellular vesicles; or

c. the method is used in combination with one or more of the following diagnostic assays:

(i) the subject's annual physical examination;

(ii) a cancer screening test;

(iii) a genetic assay to screen blood plasma for genetic mutations in circulating tumor DNA and/or protein biomarkers linked to cancer;

(iv) an assay involving immunofluorescent staining to identify cell phenotype and marker expression, followed by amplification and analysis by next-generation sequencing; and

(v) germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, and/or circulating tumor cells.

13. The method of claim 10, wherein the target biomarker signature comprises

(i) a first target biomarker comprising an extracellular vesicle-associated membrane-bound polypeptide; and

(ii) a second target biomarker comprising a target biomarker selected from the group consisting of: surface protein biomarkers, intravesicular protein biomarkers, and intravesicular RNA biomarkers.

14. The method of claim 1, further comprising performing a capture assay prior to the step (a).

15. The method of claim 14, wherein the capture assay involves contacting the sample with a capture agent comprising a target-capture moiety that binds to an extracellular vesicle-associated membrane-bound polypeptide.

16. A method of detecting cancer, the method comprising steps of:

detecting on surfaces of intact extracellular vesicles from a human blood sample co-localization of at least two biomarkers whose combined expression level has been determined to be associated with cancer;

comparing the detected co-localization level with the determined level; and

detecting cancer when the detected co-localization level is at or above the determined level.

17. The method of claim 1, wherein the cancer is selected from the group of cancers consisting of acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer, optionally wherein:

a) the cancer is colorectal cancer;

b) the cancer is lung cancer;

c) the cancer is breast cancer;

d) the cancer is ovarian cancer;

e) the step of detecting on surfaces comprises analyzing extracellular vesicles that have been separated from other components of the sample by affinity capture targeting a cancer-specific biomarker on their surfaces;

f) the step of detecting on surfaces comprises contacting the extracellular vesicles with at least one set of detection probes, each directed to a target biomarker, which set comprises at least a first detection probe for a first target biomarker and a second detection probe for a second target biomarker, for example wherein:

i) the first detection probe comprises a first target-binding moiety and a first oligonucleotide domain coupled to the first target-binding moiety, the first oligonucleotide domain comprising a first double-stranded portion and a first single-stranded overhang extended from one end of the first oligonucleotide domain; and

wherein the second detection probe comprises a second target-binding moiety and a second oligonucleotide domain coupled to the second target-binding moiety, the second oligonucleotide domain comprising a second double-stranded portion and a second single-stranded overhang extended from one end of the second oligonucleotide domain, wherein the second single-stranded overhang comprises

a nucleotide sequence complementary to at least a portion of the first single-stranded overhang and can thereby hybridize to the first single-stranded overhang, optionally wherein:

A) the first single-stranded overhang and/or the second single-stranded overhang are four nucleotides in length, for example wherein the first single-stranded overhang or the second single-stranded overhang has a nucleotide sequence of GAGT; or

B) the first oligonucleotide domain and the second oligonucleotide domain have a combined length such that, when the first and second target biomarkers are simultaneously present on the extracellular vesicles and the probes of the set of detection probes are bound to their respective target biomarkers on the extracellular vesicles, the first single-stranded overhang and the second single-stranded overhang can hybridize together, forming a double-stranded complex, for example further comprising contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template comprising a strand of the first double-stranded portion and a strand of the second double-stranded portion, for example wherein the nucleic acid ligase is or comprises a DNA ligase (e.g., T4 or T7 DNA ligase); or

ii) the first target biomarker and the second target biomarker are the same target biomarker;

g) the step of detecting cancer further comprises a step of amplifying a product that is associated with the co-localization, and detecting the presence of the amplified product, optionally wherein the step of amplifying is or comprises quantitative polymerase chain reaction; or

h) the step of detecting on surfaces comprises immobilizing extracellular vesicles on a solid substrate, optionally wherein:

i) the solid substrate is or comprises a bead, for example wherein the bead is a magnetic bead; or

ii) the solid substrate is or comprises a surface, for example wherein the surface is a capture surface of a filter, a matrix, a membrane, a plate, a tube, and/or a well.

18. A method comprising steps of:

contacting a sample comprising exosomes with a set of detection probes that specifically bind to surface biomarkers on the exosomes to detect cancer-associated exosomes in the sample with a specificity within a range of 95% to 100% and sensitivity within a range of 30% to 100%.

19. The method of claim 18, wherein:

a) the sample comprising exosomes is a human blood-derived sample;

b) specificity is evaluated relative to that of a target-binding moiety for one or more other potential target entities;

c) the set of detection probes is selected for detection of cancer such that it provides a positive predictive value greater than about 10%; or

d) a sample is determined to be positive for cancer-associated exosomes if its detection level is at or above a reference threshold level that is set to achieve a specificity within a range of 95% to 100%, optionally wherein the reference threshold is about 2-3 standard deviations above the mean detection level observed in a control sample, for example wherein the control sample is a blood-derived sample from a human subject who is not diagnosed with cancer; ore) the cancer is selected from the group of cancers consisting of acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer.

20. A method comprising steps of:

capturing exosomes from a biological sample with a capture agent that selectively interacts with a cancer-specific surface protein biomarker on the exosomes; and

contacting the captured exosomes with at least one set of at least two detection probes that each selectively interacts with a surface protein biomarker on the exosomes; and

detecting a product formed when the at least two detection probes of the set are in sufficiently close proximity, such detection indicating co-localization of the surface protein biomarkers.

21. The method of claim 20, wherein:

a) the at least one set of detection probes, collectively, detects a biomarker signature indicative of cancer;

b) the capture agent is a target-binding moiety;

c) the capture agent is an antibody, optionally wherein the antibody is coupled to a solid substrate, for example wherein:

i) the solid substrate is or comprises a bead, for example wherein the bead is a magnetic bead; or

ii) the solid substrate is or comprises a surface, optionally wherein the surface is a capture surface of a filter, a matrix, a membrane, a plate, a tube, and/or a well;

d) the step of capturing comprises immobilizing the exosomes on a solid substrate, optionally wherein:

i) the solid substrate is or comprises a bead; or

ii) the solid substrate is or comprises a surface, optionally wherein the surface is a capture surface of a filter, a matrix, a membrane, a plate, a tube, and/or a well;

e) the cancer is selected from the group of cancers consisting of acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer; or

f) the set of detection probes comprises two detection probes, optionally wherein the detection probes of the set of detection probes each comprise a target-binding moiety and an oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang extended from one end of the oligonucleotide domain.

22. A method comprising steps of:

contacting a sample comprising exosomes with a set of probes that specifically bind to surface biomarkers on the exosomes to detect cancer-associated exosomes in the sample, wherein:

(i) each probe in the set comprises a target binding moiety directed to a surface biomarker on the exosomes; and

(ii) the set comprises at least one capture probe and at least two detection probes, wherein each detection probe further comprises a detection moiety.

23. The method of claim 22, wherein:

a) at least two of the target binding moieties bind to the same epitope, optionally wherein the at least two binding moieties that bind the same epitope are the same moiety;

b) the target binding moiety of each of the detection probes binds the same epitope, optionally wherein the binding moieties that bind the same epitope are the same moiety;

c) each of the detection moieties is an oligonucleotide and the at least two detection probes include at least one pair of detection probes whose oligonucleotides hybridize to one another if and only if the detection probes are in close proximity to one another, optionally wherein the hybridization generates a ligatable nucleic acid; or

d) each probe of the set includes a target binding moiety that binds to a different epitope.

24. A method comprising steps of:

performing a proximity assay that detects a surface biomarker signature on exosomes from a human subject, the step of performing being performed a period of time after a performance of a prior assay to detect the surface biomarker signature on exosomes from the human subject; and

comparing results of the performed assay with those of the prior assay.

25. The method of claim 24, wherein:

a) presence or level of the surface biomarker signature is indicative of presence or risk of cancer;

b) the step of comparing comprises determining a change in presence or level of the surface biomarker signature;

c) the human subject is receiving or has received therapy and the change is indicative of the subject's response to the therapy, optionally wherein the human subject's response to the therapy determines whether a change in the therapy is needed;

d) the period of time is at least six months;

e) the human subject is an asymptomatic human subject;

f) the human subject has a family history of cancer;

g) the human subject has been previously treated for cancer;

h) the human subject is at risk for cancer;

i) the human subject is at risk for cancer recurrence after cancer treatment;

j) the human subject is in remission after cancer treatment;

k) the human subject was previously diagnosed to be suffering from or susceptible to cancer; or

l) the human subject is a human subject determined to be suffering from cancer,

optionally wherein the method further comprises determining whether the human subject is likely to respond to a therapy based on the comparison between results of the performed assay with those of the prior assay.

26. A method comprising steps of:

contacting exosomes with at least two detection probes, wherein each detection probe comprises

(i) a binding moiety; and

(ii) an oligonucleotide entity,

wherein the binding moiety is the same and the oligonucleotide entities complement one another.

27. The method of claim 26, wherein the step of contacting comprises contacting exosomes with at least a first detection probe and a second detection probe, optionally wherein the first detection probe comprises a first oligonucleotide entity comprising a first double-stranded portion and a first single-stranded overhang extended from one end of the oligonucleotide entity, and

the second detection probe comprises a second oligonucleotide entity comprising a second double-stranded portion and a second single-stranded overhang extended from one end of the oligonucleotide entity

for example wherein the first single-stranded overhang and/or the second single-stranded overhang are four nucleotides in length, for example wherein the first single-stranded overhang or the second single-stranded overhang has a nucleotide sequence of GAGT.

28. A complex comprising:

(a) an exosome having at least one target biomarker on its surface; and (b) a first detection probe and a second detection probe each bound to the exosome, wherein each of the first detection probe and the second detection probe comprises:

(i) a target binding moiety directed to a target biomarker expressed by the exosome; and

(ii) an oligonucleotide domain coupled to the target binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain,

wherein the single-stranded overhang portions of the first and second detection probes are hybridized to each other.

29. The complex of claim 28, wherein:

a) the first detection probe and the second detection probe are each bound to the same target biomarker;

b) the first detection probe and the second detection probe are each bound to different target biomarkers;

c) the formation of the complex is indicative of a cancer-associated exosome; or

d) the exosome was isolated from a blood sample taken from a subject, optionally wherein the subject is a human subject, for example wherein the human subject:

i) is an asymptomatic human subject;

ii) has a family history of cancer;

iii) has been previously treated for cancer;

iv) is at risk for cancer;

v) is at risk for cancer recurrence after cancer treatment;

vi) is in remission after cancer treatment;

vii) was previously diagnosed to be suffering from or susceptible to cancer; or

viii) is a human subject determined to be suffering from cancer.

30. A composition comprising extracellular vesicles from a human blood sample bound to a set of at least two probes, each of which comprises a biomarker binding moiety and an oligonucleotide domain, wherein two or more bound probes are in proximity to one

another so that their oligonucleotide domains hybridize to each other to form a ligatable hybrid.

31. The composition of claim 30, wherein:

a) the set of probes comprises 2 probes;

b) at least two of the probes of the set have the same biomarker binding moiety;

c) at least two of the probes of the set have a different biomarker binding moiety; or

d) each of the oligonucleotide domains of each of the probes of the set of probes comprises a double-stranded portion and a single-stranded overhang extended from one end of the oligonucleotide domain, optionally wherein the single-stranded overhangs are four nucleotides in length, for example wherein the single-stranded overhang of the oligonucleotide domain of one of the probes has a nucleotide sequence of GAGT.

32. A complex comprising:

(a) an exosome comprising a cancer-associated target biomarker signature; and

(b) at least a first detection probe and a second detection probe each bound to the exosome, wherein each of the detection probes comprise:

(i) a target binding moiety directed; to the target biomarker signature; and
(ii) an oligonucleotide domain coupled to the target binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang portion extended from one end of the oligonucleotide domain, wherein the single-stranded overhang portions of the detection probes are at least partially complementary.

33. A set of probes, each of which comprises:

(a) a biomarker binding moiety that specifically binds to a surface biomarker on extracellular vesicles from cancer cells; and

(b) an oligonucleotide domain,

wherein the oligonucleotide domains of probes within the set are arranged and constructed so that, when the probes are bound to their target biomarkers, their oligonucleotide domains hybridize to one another to form a ligatable hybrid only when the target biomarkers are in proximity to one another.

34. The set of probes of claim 33, wherein:

a) the set of probes comprises 2-25 probes;

b) the surface biomarker on extracellular vesicles from cancer cells is a biomarker for a cancer selected from the group of cancers consisting of acute lymphocytic leukemia, acute myeloid leukemia, bile duct cancer, bladder cancer, brain cancer, breast cancer, cervical cancer, chronic lymphocytic leukemia, chronic myeloid leukemia, colorectal cancer, endometrial cancer, esophageal cancer, gastrointestinal cancer, Hodgkin lymphoma, kidney cancer, liver cancer, lung cancer, multiple myeloma, non-Hodgkin lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, sarcomas, skin cancer, and stomach cancer;

c) the set of probes is for use in screening a subject for cancer, optionally wherein:

i) the cancer is early-stage cancer;

ii) the subject is an asymptomatic human subject, for example wherein:

A) the asymptomatic human subject has a family history of cancer;

B) the asymptomatic human subject has been previously treated for cancer;

C) the asymptomatic human subject is at risk of cancer recurrence after cancer treatment; or

D) the asymptomatic human subject is in remission after cancer treatment; or

iii) the subject was previously screened for cancer;

d) the set of probes is for use in monitoring tumor recurrence in a subject who has been treated for cancer;

e) the set of probes is for use as a companion diagnostic in combination with cancer treatment;

f) the set of probes is for use in monitoring or evaluating efficacy of a therapy administered to a subject in need thereof; or

g) the set of probes is for use in selecting a therapy for a subject in need thereof.