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(54) **Titre : COMPOSITIONS ET METHODES DE DETECTION DE CANCER DE L'OVAIRE**

(54) **Title: COMPOSITIONS AND METHODS FOR DETECTION OF OVARIAN CANCER**

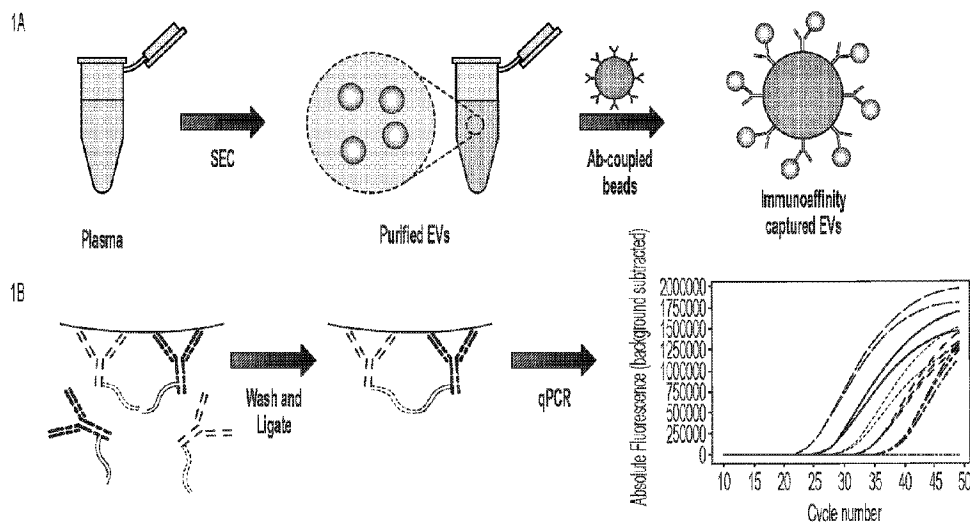


FIG. 1

(57) **Abrégé/Abstract:**

The present disclosure in one aspect provides technologies for detection of ovarian cancer, e.g., early detection of ovarian cancer. In another aspect, technologies provided herein are useful for selecting and/or monitoring and/or evaluating efficacy of, a treatment administered to a subject determined to have or susceptible to ovarian cancer. In some embodiments, technologies provided herein are useful for development of companion diagnostics, e.g., by measuring tumor burdens and changes in tumor burdens in conjunction with therapeutics. In some embodiments, technologies provided herein are useful for development of companion diagnostics, e.g., by identifying biomarkers in female subjects' bodily fluid samples (e.g., blood samples) that are associated with therapeutic response. In some embodiments, technologies provided herein are useful for differentiating a benign adnexal mass from ovarian cancer.

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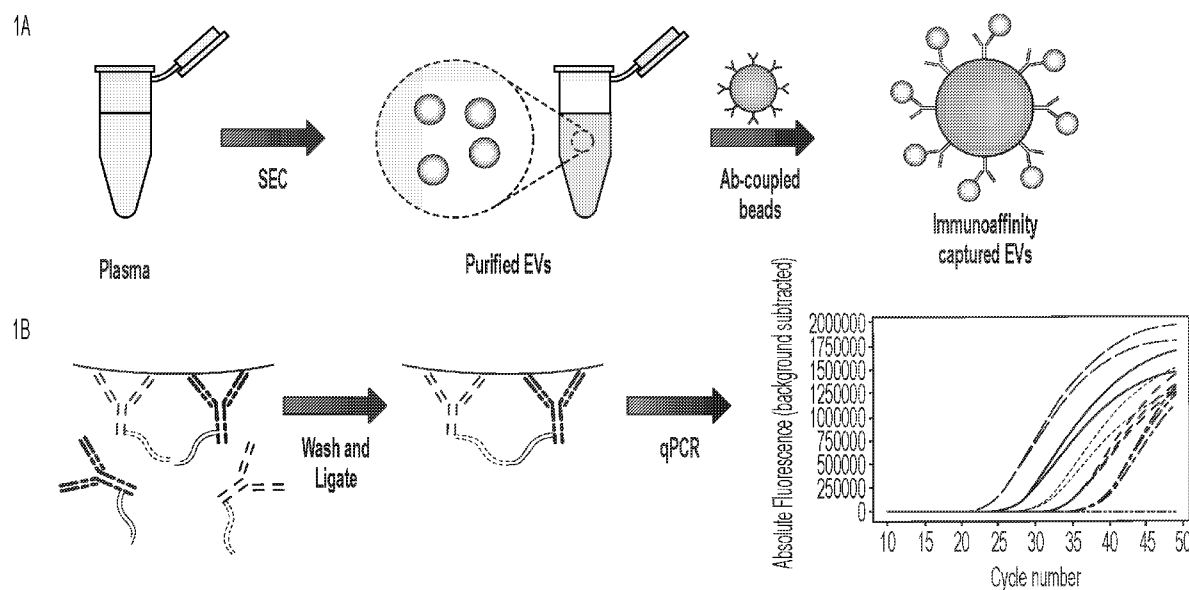


FIG. 1

(57) Abstract: The present disclosure in one aspect provides technologies for detection of ovarian cancer, *e.g.*, early detection of ovarian cancer. In another aspect, technologies provided herein are useful for selecting and/or monitoring and/or evaluating efficacy of, a treatment administered to a subject determined to have or susceptible to ovarian cancer. In some embodiments, technologies provided herein are useful for development of companion diagnostics, *e.g.*, by measuring tumor burdens and changes in tumor burdens in conjunction with therapeutics. In some embodiments, technologies provided herein are useful for development of companion diagnostics, *e.g.*, by identifying biomarkers in female subjects' bodily fluid samples (*e.g.*, blood samples) that are associated with therapeutic response. In some embodiments, technologies provided herein are useful for differentiating a benign adnexal mass from ovarian cancer.



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COMPOSITIONS AND METHODS FOR DETECTION OF OVARIAN CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 63/280603 filed November 17, 2021, U.S. Provisional Application No. 63/328250 filed April 6, 2022, and U.S. Provisional Application No. 63/417309 filed October 18, 2022, the contents of which are hereby incorporated by reference herein in their entirety.

BACKGROUND

[0002] Early detection of cancer greatly increases the chance of successful treatment. However, many cancers including ovarian cancer still lack effective screening recommendations or patient compliance with those 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

[0003] The present disclosure, among other things, provides insights and technologies for achieving effective ovarian cancer screening from a biological sample. In some embodiments, such a biological sample is or comprises a bodily fluid-derived sample, e.g., in some embodiments a blood-derived sample. In some embodiments, provided technologies are effective for detection of early-stage ovarian cancers. In some embodiments, 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 embodiments, provided technologies are effective when applied to populations comprising or consisting of individuals (*e.g.*, asymptomatic individuals) without hereditary risk in developing ovarian cancer. In some embodiments, provided technologies are effective when applied to populations comprising or consisting of symptomatic individuals (*e.g.*, individuals suffering from one or more symptoms of ovarian cancer). In some embodiments, provided technologies are effective when applied to populations comprising or consisting of individuals at risk for ovarian cancer (*e.g.*, individuals with hereditary and/or life-history associated risk factors for ovarian cancer). In some embodiments, provided technologies may be or include one or more compositions (*e.g.*, molecular entities or

complexes, systems, cells, collections, combinations, or kits) and/or methods (*e.g.*, of making, using, or assessing), as will be clear to one skilled in the art reading the disclosure provided herein.

[0004] In some embodiments, the present disclosure identifies the source of a problem with certain prior technologies including, for example, certain conventional approaches to detection and diagnosis of ovarian cancer. For example, the present disclosure appreciates that many conventional diagnostic assays, *e.g.*, based on cell-free nucleic acids, serum biomarkers (*e.g.*, CA-125, which is a portion of a MUC16 polypeptide), 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 embodiments, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by detecting co-localization of a target biomarker signature of ovarian cancer in individual nanoparticles having a size range of interest that includes extracellular vesicles, which comprises (i) at least one extracellular vesicle-associated surface biomarker and (ii) at least one target biomarker comprising one or more surface biomarkers. In some embodiments, such a target biomarker signature may further comprise one or more internal biomarkers (*e.g.*, ones described herein) and/or one or more RNA biomarkers (*e.g.*, ones described herein).

[0005] In some embodiments, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by detecting such target biomarker signature of ovarian cancer using a target entity detection approach that was developed by Applicant and described in US2020/0299780, and WO2020180741, which are based on interaction and/or co-localization of at least two or more target entities (*e.g.*, a target biomarker signature) in individual nanoparticles including, *e.g.*, extracellular vesicles.

[0006] In some embodiments, extracellular vesicles for detection as described herein can be isolated from a bodily fluid of a subject by a size exclusion-based method. As will be understood by a skilled artisan, in some embodiments, a size exclusion-based method may provide a sample comprising nanoparticles having a size range of interest that includes extracellular vesicles. Accordingly, in some embodiments, provided technologies of the present disclosure encompass detection, in individual nanoparticles having a size range of interest that includes extracellular vesicles (hereinafter “nanoparticles” as defined herein), of co-localization of at least two or more surface biomarkers (*e.g.*, as described herein) that forms a target biomarker signature of ovarian cancer. In some embodiments, such individual nanoparticles have a size range of about 30 nm to about 1000 nm. A skilled artisan reading the present disclosure will understand that various embodiments described herein in the context of “extracellular vesicle(s)” can be also applicable in the context of “nanoparticles” as described herein.

[0007] The present inventors have previously identified certain biomarker combinations and/or biomarker signatures that are useful for the detection of ovarian cancer (see, for example, WO

2121/146659). The present disclosure provides additional biomarker combinations and/or biomarker signatures that were demonstrated to achieve 90-100% specificity with certain sensitivity (*e.g.*, as described herein) when distinguishing ovarian cancer samples from reference samples (*e.g.*, normal healthy samples, benign tumor samples, and/or off-target cancer samples). In some embodiments, the present disclosure provides biomarker combinations that are particularly useful for detection of early-stage ovarian cancer, for example, with a specificity of about 90-100% and/or a sensitivity of about 80-95%. In some embodiments, the present disclosure provides biomarker combinations that are particularly useful for differentiating benign adnexal mass from ovarian cancer, for example, with a specificity of about 90-100% and/or a sensitivity of about 80-100% or about 95%-100%. In some embodiments, the present disclosure provides biomarker combinations that are particularly useful for differentiating benign adnexal mass from ovarian cancer, for example, with a positive predictive value of greater than 70% and/or a negative predictive value of greater than 98%.

[0008] In some embodiments, the present disclosure, among other things, provides insights that screening of asymptomatic 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 ovarian cancer. In some embodiments, the present disclosure provides ovarian cancer screening systems that can be implemented to detect ovarian cancer, including early-stage cancer, in some embodiments in asymptomatic individuals (*e.g.*, without hereditary risks in ovarian cancer). In some embodiments, provided technologies are implemented to achieve regular screening of asymptomatic individuals (*e.g.*, without hereditary risks in ovarian cancer). 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.*, symptomatic, or asymptomatic individuals). The present disclosure defines usefulness of such systems and provides compositions and methods for implementing them.

[0009] In some embodiments, 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 ovarian cancer, 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 embodiments, provided technologies are useful in conjunction with women's periodic physical examination such as mammogram, HPV, and/or Pap smear screening. In some embodiments, provided technologies are useful in conjunction with treatment regimen(s); in some embodiments, provided technologies may improve one or more characteristics (*e.g.*, rate of success according to an accepted parameter) of such treatment regimen(s).

[0010] In some aspects, provided are technologies for use in classifying a subject (*e.g.*, an asymptomatic subject) as having or being susceptible to ovarian cancer. In some embodiments, the

present disclosure provides methods or assays for classifying a subject (*e.g.*, an asymptomatic subject) as having or being susceptible to ovarian cancer. In some embodiments, a provided method or assay comprises (a) detecting, in a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) from a subject in need thereof, nanoparticles (having a size range of interest that includes extracellular vesicles) expressing a target biomarker signature of ovarian cancer, the target biomarker signature comprising: at least one extracellular vesicle-associated surface biomarker and at least one target biomarker comprising one or more surface biomarkers selected from (i) intact or cleaved polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof; (b) comparing sample information indicative of level of the target biomarker signature-expressing nanoparticles in the biological sample to reference information including a reference threshold level; and (c) classifying the subject as having or being susceptible to ovarian cancer when the biological sample shows an elevated level of target biomarker signature-expressing nanoparticles relative to a classification cutoff referencing the reference threshold level.

[0011] In some embodiments, at least one target biomarker comprises one or more surface biomarkers selected from (i) intact or cleaved polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0012] In some embodiments, methods or assays described herein may be performed for one more additional target biomarker signature (including, *e.g.*, at least one, at least two, at least three, or more additional target biomarker signatures). In some such embodiments, a classification cutoff may reference additional reference threshold level(s) corresponding to each additional target biomarker signature.

[0013] In some embodiments, an extracellular vesicle-associated surface biomarker for use in a target biomarker signature of ovarian cancer used and/or described herein may be or comprise a tumor-specific biomarker and/or a tissue-specific biomarker (*e.g.*, an ovarian tissue-specific biomarker). In some embodiments, such an extracellular vesicle-associated surface biomarker 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. In some embodiments, such an extracellular vesicle-associated surface biomarker may include but are not limited to (i) intact or cleaved polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9),

and combinations thereof. In some embodiments, such an extracellular vesicle-associated surface biomarker may include but are not limited to (i) intact or cleaved polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0014] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include intact or cleaved polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

[0015] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

[0016] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising an intact or cleaved polypeptide encoded by human gene *FOLR1*.

[0017] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.

[0018] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0019] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene

as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0020] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof. In some such embodiments, a surface biomarker encoded by human gene *MUC16* can be an intact MUC16 polypeptide. In some such embodiments, a surface biomarker encoded by human gene MUC16 can be a cleaved MUC16 polypeptide.

[0021] In some embodiments, a target biomarker signature for ovarian cancer detection comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene *BST2*.

[0022] In some embodiments, a reference threshold level for use in a provided method or assay described herein is determined by levels of target biomarker signature-expressing nanoparticles (having a size range of interest that includes extracellular vesicles) observed in comparable samples from a population of non-ovarian cancer subjects.

[0023] In some embodiments, an extracellular vesicle-associated surface biomarker included in a target biomarker signature may be detected using affinity agents (*e.g.*, but not limited to antibody-based agents). In some embodiments, an extracellular vesicle-associated surface biomarker may be detected using a capture assay comprising an antibody-based agent. For example, in some embodiments, a capture assay for detecting the presence of an extracellular vesicle-associated surface biomarker in an extracellular vesicle may involve contacting a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) comprising nanoparticles with a capture agent directed to such an extracellular vesicle-associated surface biomarker. In some embodiments, such a capture agent may comprise a binding moiety directed to an extracellular vesicle-associated surface biomarker (*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 surface biomarker may be or comprising a solid substrate (*e.g.*, a magnetic bead) and a binding moiety (*e.g.*, an antibody agent) directed to an extracellular vesicle-associated surface biomarker.

[0024] In some embodiments, 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 analytes *vs.* intravesicular analytes; and/or polypeptides and/or

glycoforms *vs.* carbohydrates *vs.* RNAs). For example, a person skilled in the art, reading the present disclosure, will appreciate that a surface biomarker and/or an intravesicular biomarker may be detected using affinity agents (*e.g.*, antibody-based agents) in some embodiments, while in some embodiments, an intravesicular RNA biomarker, *e.g.*, mRNA, small nuclear RNA (snRNA) microRNA (miRNA), small interfering RNA (siRNA), orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA, may be detected using nucleic acid-based agents, *e.g.*, using quantitative reverse transcription PCR.

[0025] For example, in some embodiments where a target biomarker is or comprises a surface biomarker and/or an intravesicular biomarker, 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 nanoparticles that display an extracellular vesicle-associated surface biomarker (*e.g.*, ones as used and/or described herein). In some embodiments, such a proximity ligation assay may comprise contacting a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) comprising nanoparticles 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 nanoparticles and the set of detection probes is generated, wherein the two detection probes each comprise: (i) a binding moiety directed to a surface biomarker and/or an intravesicular 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 nanoparticles 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 nanoparticles 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 nanoparticles that are positive for a target biomarker signature of ovarian 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.

[0026] In some embodiments where a target biomarker is or comprises an intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) marker, such a target biomarker may be detected involving a nucleic acid

detection assay. In some embodiments, an exemplary nucleic acid detection assay may be or comprise reverse-transcription PCR.

[0027] In some embodiments where a target biomarker is or comprises an intravesicular biomarker and/or an intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) 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 such biomarker(s) within nanoparticles for subsequent detection.

[0028] The present disclosure, among other things, recognizes that detection of a single ovarian cancer-associated serum protein or a plurality of ovarian 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 ovarian 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 nanoparticles having a size range of interest that includes extracellular vesicles for detection be characterized by presence of a target biomarker signature comprising a combination of at least one or more extracellular vesicle-associated surface biomarkers and at least one or more target biomarkers comprising one or more surface biomarkers (*e.g.*, as described herein). In particular embodiments, the present disclosure teaches technologies that require such individual nanoparticles be characterized by presence (*e.g.*, by expression) of such a target biomarker signature of ovarian cancer, while nanoparticles that do not comprise the 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 embodiments, a reference level may be a level observed in a negative control sample, such as a sample in which individual nanoparticles comprising such a target biomarker signature are absent).

[0029] Accordingly, in some embodiments, technologies provided herein can be useful for detection of incidence or recurrence of ovarian cancer in a subject and/or across a population of subjects. In some embodiments, a target biomarker signature may be selected for detection of ovarian cancer. In some embodiments, a target biomarker signature may be selected for detection of a specific category of ovarian cancer, including, *e.g.*, but not limited to high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, and/or mucinous ovarian cancer. In some embodiments, 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 ovarian cancer or ovarian cancer recurrence.

[0030] In some embodiments, a subject that is amenable to technologies provided herein for detection of incidence or recurrence of ovarian 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 ovarian cancer, who has a life history which places them him/her at increased risk for ovarian cancer, who is post-menopausal, who has been previously treated for ovarian cancer, who is at risk of ovarian cancer recurrence after cancer treatment, who is in remission after ovarian cancer treatment, and/or who has been previously or periodically screened for the presence of at least one ovarian cancer biomarker, *e.g.*, but not limited to CA-125 plasma proteins. In some embodiments, such an asymptomatic subject may be a subject who is determined to have a normal plasma CA-125 level (*e.g.*, a plasma CA-125 level of less than 35 U/mL). In some embodiments, such an asymptomatic subject may be a subject who is determined to have a plasma CA-125 level of equal to or higher than a normal plasma CA-125 level. Alternatively, in some embodiments, an asymptomatic subject may be a subject who has not been previously screened for ovarian cancer, who has not been diagnosed for ovarian cancer, and/or who has not previously received ovarian cancer therapy.

[0031] In some embodiments, a subject or population of subjects may be selected based on one or more characteristics such as age, race, geographic location, genetic history, personal and/or medical history (*e.g.*, smoking, alcohol, drugs, carcinogenic agents, diet, obesity, diabetes, physical activity, sun exposure, radiation exposure, perineal talc use, hormone replacement therapy (HRT), exposure to infectious agents such as viruses, and/or occupational hazard).

[0032] In some embodiments, technologies provided herein can be useful for selecting surgery or therapy for a subject who is suffering from or susceptible to ovarian cancer. In some embodiments, an ovarian cancer surgery, therapy and/or an adjunct therapy can be selected in light of findings based on technologies provided herein.

[0033] In some embodiments, technologies provided herein can be useful for monitoring and/or evaluating efficacy of therapy administered to a subject (*e.g.*, an ovarian cancer subject).

[0034] In some embodiments, 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 embodiments, 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 embodiments, 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 older than a certain age (*e.g.*, over 40, 45, 50, 55, 60, 65, 70, or older). In some embodiments, 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 ovarian cancer as described herein. For example, in

some embodiments, 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 ovarian cancer), identification of one or more risk factors associated with ovarian cancer (*e.g.*, life history risk factors including, *e.g.*, but not limited to 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 ovarian cancer (*e.g.*, abnormal bleeding in-between a woman's period potentially indicative of ovarian cancer, *etc.*).

[0035] In some embodiments, 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 embodiments, provided technologies can provide determination of whether individual subjects have one or more indicators of incidence or recurrence of ovarian cancer, thereby informing physicians and/or patients when to initiate therapy in light of such findings. Additionally, or alternatively, in some embodiments, provided technologies can inform physicians and/or patients of treatment selection, *e.g.*, based on findings of specific responsiveness biomarkers (*e.g.*, ovarian cancer responsiveness biomarkers). In some embodiments, 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 ovarian 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.

[0036] In some embodiments, 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 embodiments, 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 embodiments, 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.

[0037] In some embodiments, provided technologies can aid in the diagnosis of ovarian cancer in symptomatic individuals with an imaging-confirmed adnexal mass. In some such embodiments, a positive test result is interpreted in conjunction with other clinical findings to diagnose cancer. In some embodiments, such clinical findings to diagnose cancer may include, for example, pelvic or abdominal pain, inability to eat or feeling "full," and/or increased abdominal size or bloating, and other clinical findings as described, for example, for example, in Goff *et al.*, Development of an ovarian cancer symptom index. *Cancer*. 2007;109: 221-227, the entire content of which is incorporated herein by reference for the purposes described herein.

[0038] Some aspects provided herein relate to systems and kits for use in provided technologies. In some embodiments, a system or kit may comprise detection agents for a tumor biomarker signature of ovarian cancer (*e.g.*, ones described herein). In some embodiments, such a system or kit may comprise a capture agent for an extracellular vesicle-associated surface biomarker present in nanoparticles associated with ovarian cancer (*e.g.*, ones used and/or described herein); and (b) at least one or more detection agents directed to one or more target biomarkers of a target biomarker signature of ovarian cancer, which may be or comprise additional surface biomarker(s) (*e.g.*, ones as used and/or described herein). In some embodiments, such a system or kit may further comprise one or more detection agents directed to intravesicular biomarker(s) (*e.g.*, ones as used and/or described herein), and/or intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) biomarker(s) (*e.g.*, ones as used and/or described herein), which are determined to be useful for ovarian cancer detection.

[0039] In some embodiments, a capture agent included in a system and/or kit may comprise a binding moiety directed to an extracellular vesicle-associated surface biomarker (*e.g.*, ones described herein). In some embodiments, such a binding moiety may be conjugated to a solid substrate, which in some embodiments may be or comprise a solid substrate. In some embodiments, such a solid substrate may be or comprise a magnetic bead. In some embodiments, 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 affinity reagent (*e.g.*, but not limited to an antibody agent) directed to an extracellular vesicle-associated surface biomarker conjugated thereto.

[0040] In some embodiments where a target biomarker includes a surface biomarker and/or an intravesicular biomarker, a system and/or kit may include detection agents for performing a proximity ligation assay (*e.g.*, ones as described herein). In some embodiments, 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.

[0041] In some embodiments, 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 embodiments, at least one set of detection probes may be directed to detection for ovarian cancer. For example, in some embodiments, a provided system and/or kit may comprise at least one set for detection probes for detection of ovarian cancer and at least one set of detection probes for detection of a different cancer (*e.g.*, pancreatic cancer). In some embodiments, two or more detection probes may be directed to different categories of ovarian cancer, *e.g.*, high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer. In some embodiments, two or more sets may be directed to detection of ovarian cancer of different stages. In some embodiments, two or more sets may be directed to detection of ovarian cancer of the same stage.

[0042] In some embodiments, detection probes in a provided kit may be provided as a single mixture in a container. In some embodiments, multiple sets of detection probes may be provided as individual mixtures in separate containers. In some embodiments, each detection probe is provided individually in a separate container.

[0043] In some embodiments where a target biomarker includes an intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) biomarker, such a system and/or kit may include detection agents for performing a nucleic acid detection assay. In some embodiments, 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, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) target(s).

[0044] In some embodiments, a provided system and/or kit may comprise at least one chemical reagent, *e.g.*, to process a sample and/or nanoparticles therein. In some embodiments, a provided system and/or kit may comprise at least one chemical reagent to process nanoparticles in a sample, including, *e.g.*, but not limited to a fixation agent, a permeabilization agent, and/or a blocking agent. In some embodiments, a provided system and/or kit may comprise a nucleic acid ligase and/or a nucleic acid polymerase. In some embodiments, a provided system and/or kit may comprise one or more primers and/or probes. In some embodiments, 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 embodiments, a provided system and/or kit may comprise one or more probes such as, for example, hydrolysis probes which may in some embodiments be designed to increase the specificity of qPCR (*e.g.*, TaqMan probes). In some embodiments, 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).

[0045] In some embodiments, 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 ovarian cancer. In some embodiments, a provided system and/or kit can be used for screening and/or other assessment of individuals susceptible to ovarian cancer (*e.g.*, individuals with a known genetic, environmental, or experiential risk, *etc.*). In some embodiments, provided system and/or kits can be used for monitoring recurrence of ovarian cancer in a subject who has been previously treated. In some embodiments, provided systems and/or kits can be used as a companion diagnostic in combination with a therapy for a subject who is suffering from ovarian cancer. In some embodiments, provided systems and/or kits can be used for monitoring or evaluating efficacy of a therapy administered to a subject who is suffering from ovarian cancer. In some embodiments, provided systems and/or kits can be used for selecting a therapy for a subject who is suffering from ovarian cancer. In some embodiments, 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 ovarian cancer.

[0046] 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 embodiments, a complex comprises: an extracellular vesicle expressing a target biomarker signature, which includes at least one extracellular vesicle-associated surface biomarker and at least one target biomarker comprising one or more surface biomarkers (*e.g.*, described herein), wherein the extracellular vesicle is immobilized onto a solid substrate comprising a binding moiety directed to such a extracellular vesicle-associated surface biomarker. In some embodiments, such a complex further comprises at least two detection probes directed to at least one target biomarker of a target biomarker signature present in the extracellular vesicle, wherein each detection probe is bound to a respective 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.

[0047] In some embodiments, an extracellular vesicle-associated surface biomarker present in an extracellular vesicle that forms a complex may comprise one or more of polypeptides encoded

by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0048] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include intact or cleaved polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

[0049] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

[0050] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising an intact or cleaved polypeptide encoded by human gene *FOLR1*.

[0051] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.

[0052] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0053] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises an intact or cleaved polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded

by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0054] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof. In some such embodiments, a surface biomarker encoded by human gene *MUC16* can be an intact MUC16 polypeptide. In some such embodiments, a surface biomarker encoded by human gene *MUC16* can be a cleaved MUC16 polypeptide.

[0055] In some embodiments, a target biomarker signature expressed by ovarian cancer-associated nanoparticles comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one intact or cleaved polypeptide encoded by human gene *BST2*.

[0056] One aspect of the disclosure herein is a method comprising steps of:

- a) providing or obtaining a biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) from a subject;
- b) detecting, in the biological sample, nanoparticles expressing a first target biomarker signature ("first target biomarker signature-expressing nanoparticles"), the first target biomarker signature comprising:
 - i) at least one extracellular vesicle-associated surface biomarker and
 - ii) at least one target biomarker selected from surface biomarkers, wherein:
 - iii) the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof;
- c) comparing sample information indicative of level of the first target biomarker signature-expressing nanoparticles in the biological sample to reference information including a first reference threshold level;
- d) classifying the subject as having or being susceptible to ovarian cancer when the biological sample shows an elevated level of first target biomarker signature-expressing nanoparticles relative to a classification cutoff referencing the first reference threshold level.

[0057] In some embodiments of the disclosed method, when the surface biomarker is a polypeptide encoded by the human gene *MUC16*, the polypeptide an intact MUC16 polypeptide.

[0058] In some embodiments of the disclosed method, when the surface biomarker is a polypeptide encoded by the human gene *MUC16*, the polypeptide a cleaved MUC16 polypeptide.

[0059] In some embodiments of the disclosed method, the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.

[0060] In some embodiments of the disclosed method, when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different.

[0061] In some embodiments of the disclosed method, the steps of (b) and (c) are repeated for at least a second target biomarker signature, and wherein the classification cutoff references the first reference threshold level and at least a second reference threshold level corresponding to the at least a second target biomarker signature.

[0062] In some embodiments of the disclosed method, the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0063] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises at least one extracellular vesicle-associated surface biomarker and at least two biomarkers selected from the group consisting of: surface biomarkers, intravesicular biomarkers, and intravesicular RNA biomarkers.

[0064] In some embodiments of the disclosed method, the at least two biomarkers comprise one of the following combinations:

- a) - at least two distinct surface biomarkers;
- b) - at least two distinct intravesicular biomarkers;
- c) - at least two distinct intravesicular RNA biomarkers;
- d) - a surface biomarker and an intravesicular biomarker;
- e) - a surface biomarker and an intravesicular RNA biomarker; and
- f) - an intravesicular biomarker and an intravesicular RNA biomarker.

[0065] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

[0066] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target

surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

[0067] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.

[0068] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.

[0069] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0070] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0071] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof.

[0072] In some embodiments of the disclosed method, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide.

[0073] In some embodiments of the disclosed method, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.

[0074] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.

[0075] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.

[0076] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker *BST2*.

[0077] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least two target biomarkers, which are *BST2* and *FOLR1*.

[0078] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least one target biomarker sTn antigen.

[0079] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *BST2* and MUC1.

[0080] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *FOLR1* and MUC1.

[0081] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and MSLN.

[0082] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are *FOLR1* and MUC16.

[0083] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker,

which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.

[0084] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.

[0085] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.

[0086] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.

[0087] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.

[0088] In some embodiments of the disclosed method, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.

[0089] In some embodiments of the disclosed method, the first or second reference threshold level is determined by levels of target biomarker signature-expressing nanoparticles observed in comparable samples from a population of non-cancer subjects.

[0090] In some embodiments of the disclosed method, the population of non-cancer subjects comprises one or more of the following subject populations: healthy subjects, subjects diagnosed with benign tumors, and subjects with non-ovarian-related diseases, disorders, and/or conditions.

[0091] In some embodiments of the disclosed method, the biological sample has been subjected to purification (*e.g.*, size exclusion chromatography) to isolate (*e.g.*, directly from the biological sample) nanoparticles having a size range of interest that includes nanoparticles.

[0092] In some embodiments of the disclosed method, the step of detecting comprises a capture assay.

[0093] In some embodiments of the disclosed method, the capture assay involves contacting the biological sample with a capture agent comprising a target-capture moiety that binds to the at least one extracellular vesicle-associated surface biomarker.

[0094] In some embodiments of the disclosed method, the capture agent is or comprises a solid substrate comprising the target-capture moiety conjugated thereto. In some embodiments, the solid substrate comprises a magnetic bead.

[0095] In some embodiments of the disclosed method, the target-capture moiety is or comprises an antibody agent.

[0096] In some embodiments of the disclosed method, the step of detecting comprises a detection assay.

[0097] In some embodiments of the disclosed method, the step of detecting comprises a capture assay and a detection assay, the capture assay being performed prior to the detection assay.

[0098] In some embodiments of the disclosed method, when the first and/or second target biomarker signature comprises at least one intravesicular RNA biomarkers, the detection assay involves reverse transcription qPCR.

[0099] In some embodiments of the disclosed method, when the first and/or second target biomarker signature comprises at least one intravesicular biomarker, the target biomarker signature-expressing nanoparticles are processed involving fixation and/or permeabilization prior to the detection assay.

[0100] In some embodiments of the disclosed method, when the first and/or second target biomarker signature comprises at least one surface biomarker and/or intravesicular biomarker, the detection assay involves an immunoassay (including, *e.g.*, immuno-PCR, and/or proximity ligation assay).

[0101] In some embodiments of the disclosed method, the detection assay involves a proximity ligation assay. In some embodiments, the proximity ligation assay comprises the steps of:

- a) contacting the target biomarker signature-expressing nanoparticles that express the at least one extracellular vesicle-associated surface biomarker (“extracellular vesicle-associated surface biomarker-expressing nanoparticles”) with a set of detection probes, each directed to a target biomarker of the target biomarker signature, which set comprises at least two detection probes, so that a combination comprising the nanoparticles and the set of detection probes is generated, wherein the detection probes each comprise:
 - i) a target binding moiety directed to the target biomarker of 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle,

- b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the nanoparticles such that the at least two detection probes can bind to the same extracellular vesicle that express the target biomarker signature to form a double-stranded complex;
- c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template; and
- d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the biological sample of the target biomarker signature-expressing nanoparticles; and
- e) optionally repeating steps a through d at least one additional time using an orthogonal target biomarker signature.

[0102] In some embodiments, the target binding moiety of the at least two detection probes are directed to the same target biomarker. In some embodiments, the oligonucleotide domain of the at least two detection probes are different.

[0103] In some embodiments of the disclosed method, the target-capture moiety of the capture assay is or comprises at least one antibody agent directed to the at least one extracellular vesicle-associated surface biomarker.

[0104] In some embodiments of the disclosed method, the method is performed to screen for early-stage ovarian cancer, late-stage ovarian cancer, or recurrent ovarian cancer in the subject.

[0105] In some embodiments of the disclosed method, the subject is determined to have a normal plasma CA-125 level.

[0106] In some embodiments of the disclosed method, the subject has at least one or more of the following characteristics:

- a) an asymptomatic female (*e.g.*, woman) who is susceptible to ovarian cancer (*e.g.*, at an average population risk (*i.e.*, without hereditary risk) or with hereditary risk for ovarian cancer);
- b) a post-menopausal woman;
- c) a female (*e.g.*, woman) with a family history of breast and/or ovarian cancer (*e.g.*, a female (*e.g.*, woman) having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer);
- d) a female (*e.g.*, woman) determined to have one or more germline mutations in ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11, TP53, BARD, CHEK2, MRE11A, RAD50, RAD51C, RAD51D and combinations thereof;

- e) a female (*e.g.*, woman) with breast cancer determined to have germline mutations in BRCA1, BRCA2 and/or PALB2;
- f) an elderly woman *e.g.*, age 65 or above;
- g) a female (*e.g.*, woman) with one or more non-specific symptoms of ovarian cancer, optionally wherein at least one of the non-specific symptoms is similar to one or more symptoms for irritable bowel syndrome; and
- h) a female (*e.g.*, woman) recommended for CA-125/transvaginal ultrasound (TVUS) periodic screening;
- i) a female (*e.g.*, woman) diagnosed with an imaging-confirmed adnexal mass;
- j) a female (*e.g.*, woman) at hereditary risk before undergoing a risk-reducing bilateral salpingo-oophorectomy;
- k) a female (*e.g.*, woman) with a benign gynecological tumor;
- l) a female (*e.g.*, woman) who has been previously treated for ovarian cancer; and
- m) a female (*e.g.*, woman) with life-history associated risk for ovarian cancer.

[0107] In some embodiments of the disclosed method, the method is used in combination with one or more of the following diagnostic assays:

- a) the subject's annual physical examination (*e.g.*, including a HPV, and/or Pap smear screening for cervical cancer and a mammogram screening for breast cancer).
- b) plasma CA-125 and/or TVUS screening test;
- c) a genetic assay to screen blood plasma for genetic mutations in circulating tumor DNA and/or protein biomarkers linked to cancer;
- d) an assay involving immunofluorescent staining to identify cell phenotype and marker expression, followed by amplification and analysis by next-generation sequencing; and
- e) BRCA1 and/or BRCA2 germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, OVA1 and OVERA tests, and/or circulating tumor cells.

[0108] In some embodiments of the disclosed method, the ovarian cancer is high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer.

[0109] In some embodiments of the disclosed method, the ovarian cancer is high-grade serous ovarian cancer. In some embodiments, the high-grade serous ovarian cancer is at an early stage.

[0110] In some embodiments, the disclosed method is performed to monitor an ovarian cancer patient for response to treatment of an anti-ovarian cancer therapy (*e.g.*, olaparib, cisplatin, rucaparib, niraparib, talazoparib) and/or for cancer recurrence/metastasis.

[0111] In some embodiments, the disclosed method comprises steps of: detecting on surfaces of intact nanoparticles 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.

[0112] In some embodiments, the disclosed method comprises 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%.

[0113] In some embodiments, the disclosed method comprises steps of: capturing exosomes from a biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) with a capture agent that selectively interacts with a cancer-specific surface 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 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 biomarkers.

[0114] In some embodiments, the disclosed method comprises 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.

[0115] In some embodiments, the disclosed method comprises 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.

[0116] In some embodiments, the disclosed method comprises 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.

[0117] In some embodiments, the disclosed method comprises detecting marker proximity on exosome surfaces, including an improvement that comprises contacting the exosomes with at least a pair of binding agents that each comprise a binding moiety and a proximity moiety, wherein the binding moieties are the same and the proximity moieties complement one another; and detecting an interaction between the proximity moieties.

[0118] One aspect of the disclosure herein is a kit for detection of ovarian cancer comprising:

- a) a capture agent comprising a target-capture moiety directed to an extracellular vesicle-associated surface biomarker; and
- b) at least one set of detection probes, which set comprises at least two detection probes each directed to a target biomarker of a target biomarker signature for ovarian cancer, wherein the detection probes each comprise:
 - i) a target binding moiety directed at the target biomarker of the target biomarker signature for ovarian cancer; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle;

wherein the target biomarker signature for ovarian cancer comprises:

at least one extracellular vesicle-associated surface biomarker and

at least one target biomarker selected from surface biomarkers, wherein:

the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*;

and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0119] In some embodiments of the disclosed kit, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact *MUC16* polypeptide.

[0120] In some embodiments of the disclosed kit, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved *MUC16* polypeptide.

[0121] In some embodiments of the disclosed kit, the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.

[0122] In some embodiments of the disclosed kit, when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different.

[0123] In some embodiments of the disclosed kit, the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn)

antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0124] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

[0125] In some embodiments of the disclosed kit, the the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

[0126] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.

[0127] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.

[0128] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0129] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0130] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or

comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof. In some embodiments, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide. In some embodiments, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.

[0131] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.

[0132] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.

[0133] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker *BST2*.

[0134] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least two target biomarkers, which are *BST2* and *FOLR1*.

[0135] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least one target biomarker sTn antigen.

[0136] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *BST2* and *MUC1*.

[0137] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *FOLR1* and *MUC1*.

[0138] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *MUC16* and *MSLN*.

[0139] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or

comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are FOLR1 and MUC16.

[0140] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.

[0141] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.

[0142] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.

[0143] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.

[0144] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.

[0145] In some embodiments of the disclosed kit, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.

[0146] In some embodiments of the disclosed kit, the target binding moiety of the at least two detection probes is each directed to the same target biomarker of the target biomarker signature.

[0147] In some embodiments of the disclosed kit, the oligonucleotide domain of the at least two detection probes are different.

[0148] In some embodiments of the disclosed kit, the target binding moiety of the at least two detection probes is each directed to a distinct target biomarker of the target biomarker signature.

[0149] In some embodiments, the disclosed kit further comprises at least one additional reagent (*e.g.*, a ligase, a fixation agent, and/or a permeabilization agent).

[0150] In some embodiments, the disclosed kit comprises at least two sets (including, *e.g.*, at least three sets) of detection probes, which each set comprises at least two detection probes each directed to a target biomarker of a distinct target biomarker signature for ovarian cancer.

[0151] In some embodiments, the disclosed kit comprises:

- a) a first capture agent comprising a target-capture moiety;
- b) a second capture agent comprising a target-capture moiety;

- c) at least two sets of detection probes, wherein the detection probes each comprise:
 - i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle.

[0152] In some embodiments, the disclosed kit comprises:

- a) a first capture agent comprising a target-capture moiety;
- b) a second capture agent comprising a target-capture moiety;
- c) a third capture agent comprising a target-capture moiety;
- d) at least three sets of detection probes, wherein the detection probes each comprise:
 - i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle.

[0153] One aspect of the disclosure herein is a complex comprising:

- a) an extracellular vesicle expressing a target biomarker signature for ovarian cancer, wherein the target biomarker signature comprises:
 - at least one extracellular vesicle-associated surface biomarker and
 - at least one target biomarker selected from surface biomarkers, wherein:
 - the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof;
 - wherein the extracellular vesicle is immobilized onto a solid substrate comprising a target-capture moiety directed to the extracellular vesicle-associated surface biomarker;
- b) a first detection probe and a second detection probe each bound to the extracellular vesicle, wherein each detection probe comprises:
 - i) a target binding moiety directed to one of the target biomarker of the tumor 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 first and second detection probes are hybridized to each other.

[0154] In some embodiments of the disclosed complex, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact *MUC16* polypeptide.

[0155] In some embodiments of the disclosed complex, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved *MUC16* polypeptide.

[0156] In some embodiments of the disclosed complex, the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.

[0157] In some embodiments of the disclosed complex, when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different;

[0158] In some embodiments of the disclosed complex, the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0159] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

[0160] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

[0161] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.

[0162] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.

[0163] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0164] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

[0165] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof. In some embodiments, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide. In some embodiments, the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.

[0166] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.

[0167] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.

[0168] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BST2.

[0169] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least two target biomarkers, which are BST2 and FOLR1.

[0170] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least one target biomarker sTn antigen.

[0171] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are BST2 and MUC1.

[0172] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are FOLR1 and MUC1.

[0173] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and MSLN.

[0174] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are FOLR1 and MUC16.

[0175] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.

[0176] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.

[0177] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.

[0178] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.

[0179] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.

[0180] In some embodiments of the disclosed complex, the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.

[0181] In some embodiments of the disclosed complex, the target binding moiety of the at least two detection probes is each directed to the same target biomarker of the target biomarker signature. In some embodiments, the oligonucleotide domain of the at least two detection probes are different.

[0182] In some embodiments of the disclosed complex, the target binding moiety of the at least two detection probes is each directed to a distinct target biomarker of the target biomarker signature.

[0183] In some embodiments of the disclosed complex, the solid substrate comprises a magnetic bead.

[0184] In some embodiments of the disclosed complex, the target-capture moiety is or comprises an antibody agent.

[0185] In some embodiments, the disclosed complex comprises (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.

[0186] In some embodiments, the disclosed complex comprises nanoparticles 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.

[0187] In some embodiments, the disclosed complex comprises (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.

[0188] One aspect of the disclosure herein is a set of probes for use in a method, kit, or complex, wherein each set of probes comprises: (a) a biomarker binding moiety that specifically binds to a surface biomarker on nanoparticles 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.

[0189] One aspect of the disclosure herein is a method comprising steps of:

- a) providing or obtaining a biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) from a female subject;
- b) detecting, in the biological sample, nanoparticles expressing a first target biomarker signature (“first target biomarker signature-expressing nanoparticles”), the first target biomarker signature comprising:
 - i) at least one extracellular vesicle-associated surface biomarker; and
 - ii) at least one target surface biomarker,
 wherein the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker are each independently selected from:
 - (1) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; and
 - (2) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (3) combinations thereof;
- c) comparing sample information indicative of level of the first target biomarker signature-expressing nanoparticles in the biological sample to reference information including a first reference threshold level;
- d) classifying the subject as having or being susceptible to ovarian cancer when the biological sample shows an elevated level of the first target biomarker signature-expressing nanoparticles relative to a classification cutoff referencing the first reference threshold level.

[0190] In some embodiments of the disclosed method, the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker are different.

[0191] In some embodiments of the disclosed method, the steps of (b) and (c) are repeated for at least a second target biomarker signature, and wherein the classification cutoff references the first reference threshold level and at least a second reference threshold level corresponding to the at least a second target biomarker signature.

[0192] In some embodiments of the disclosed method, the steps of (b) and (c) are repeated for a plurality of additional target biomarker signatures, and wherein the classification cutoff references each reference threshold level corresponding to each target biomarker signature.

[0193] In some embodiments of the disclosed method, the first target biomarker signature or at least one of the target biomarker signatures comprises at least one extracellular vesicle-associated surface biomarker and at least one target surface biomarker, which combination is selected from the following:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*; and
- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.

[0194] In some embodiments of the disclosed method, the first target biomarker signature or at least one of the target biomarker signatures comprises at least one extracellular vesicle-associated surface biomarker and at least two target surface biomarkers, which combination is selected from the following:

- a) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- b) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- c) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- d) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0195] In some embodiments of the disclosed method, the first target biomarker signature and the plurality of additional target biomarker signatures collectively comprise the following combinations of the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;

- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- d) (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- e) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- f) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- g) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0196] In some embodiments of the disclosed method, the reference threshold level(s) is/are determined by level(s) of the corresponding target biomarker signature-expressing nanoparticles observed in comparable samples from a population of non-cancer subjects.

[0197] In some embodiments of the disclosed method, the population of non-cancer subjects comprises one or more of the following subject populations: healthy subjects, subjects diagnosed with benign tumors, and subjects with non-ovarian-related diseases, disorders, and/or conditions.

[0198] In some embodiments of the disclosed method, the biological sample has been subjected to purification (*e.g.*, size exclusion chromatography) to isolate (*e.g.*, directly from the biological sample) nanoparticles having a size range of interest that includes nanoparticles.

[0199] In some embodiments of the disclosed method, the step of detecting comprises a capture assay. In some embodiments, the capture assay involves contacting the biological sample with a capture probe comprising a target-capture moiety that binds to the at least one extracellular vesicle-associated surface biomarker. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to the at least one extracellular vesicle-associated surface biomarker. In some embodiments, the at least one extracellular vesicle-associated surface biomarker is or comprises a sialyl Lewis A antigen (also known as CA19-9), a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *MUC16*, or a sialyl Tn (sTn) antigen. In some embodiments, the capture probe is or comprises a solid substrate comprising the target-capture moiety conjugated thereto. In some embodiments, the solid substrate comprises a magnetic bead.

[0200] In some embodiments of the disclosed method, the step of detecting comprises a detection assay. In some embodiments, the step of detecting comprises a capture assay and a detection assay, the capture assay being performed prior to the detection assay. In some embodiments, the detection assay involves an immunoassay (including, *e.g.*, immuno-PCR, and/or proximity ligation assay). In some embodiments, the detection assay involves a proximity ligation assay. In some embodiments, the proximity ligation assay comprises the steps of:

- a) contacting nanoparticles in the biological sample with a set of detection probes, each directed to the at least one target surface biomarker of the target biomarker signature, which set comprises at least two detection probes, so that a complex comprising the nanoparticles and the set of detection probes is generated, wherein the detection probes each comprise:
 - i) a target binding moiety directed to one of the at least one target surface biomarker of 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle,
- b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the nanoparticles such that the at least two detection probes can bind to the same extracellular vesicle that express the target biomarker signature to form a double-stranded complex;
- c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template;
- d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the biological sample of the target biomarker signature-expressing nanoparticles; and
- e) optionally repeating steps (a) through (d) at least one additional time using an orthogonal target biomarker signature.

[0201] In some embodiments, the target binding moieties of the at least two detection probes are each directed to the same target surface biomarker. In some embodiments, the oligonucleotide domain of the at least two detection probes are different. In some embodiments, the same target surface biomarker is or comprises a polypeptide encoded by human gene *BST2*. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the same target surface biomarker is or comprises a sialyl Tn (sTn) antigen. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*. In some embodiments of the disclosed method, the target binding moieties of the at least two detection probes are each directed to a distinct target surface biomarker. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide

encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*. In some embodiments, the target-capture moiety of the capture assay is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.

[0202] In some embodiments of the disclosed method, the method is performed to screen for early-stage ovarian cancer, late-stage ovarian cancer, or recurrent ovarian cancer in the subject.

[0203] In some embodiments of the disclosed method, the method is performed to screen for early-stage ovarian cancer.

[0204] In some embodiments of the disclosed method, the subject has at least one or more of the following characteristics:

- a) an asymptomatic female (*e.g.*, woman) who is susceptible to ovarian cancer (*e.g.*, at an average population risk (*i.e.*, without hereditary risk) or with hereditary risk for ovarian cancer);
- b) a post-menopausal woman;
- c) a female (*e.g.*, woman) with a family history of breast and/or ovarian cancer (*e.g.*, a female (*e.g.*, woman) having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer);
- d) a female (*e.g.*, woman) determined to have one or more germline mutations in ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11, TP53, BARD, CHEK2, MRE11A, RAD50, RAD51C, RAD51D and combinations thereof;
- e) a female (*e.g.*, woman) with breast cancer determined to have germline mutations in BRCA1, BRCA2 and/or PALB2;
- f) an elderly woman *e.g.*, age 65 or above;
- g) a female (*e.g.*, woman) with one or more non-specific symptoms of ovarian cancer, optionally wherein at least one of the non-specific symptoms is similar to one or more symptoms for irritable bowel syndrome; and

- h) a female (*e.g.*, woman) recommended for plasma CA-125/transvaginal ultrasound (TVUS) periodic screening;
- i) a female (*e.g.*, woman) diagnosed with an imaging-confirmed adnexal mass;
- j) a female (*e.g.*, woman) at hereditary risk for ovarian cancer before undergoing a risk-reducing bilateral salpingo-oophorectomy;
- k) a female (*e.g.*, woman) with a benign gynecological tumor;
- l) a female (*e.g.*, woman) who has been previously treated for ovarian cancer; and
- m) a female (*e.g.*, woman) with life-history associated risk for ovarian cancer.

[0205] In some embodiments of the disclosed method, the subject is determined to have a normal serum CA-125 level (*e.g.*, equal to or lower than 25 U/mL).

[0206] In some embodiments of the disclosed method, the female subject is diagnosed with an imaging-confirmed adnexal mass. In some embodiments, the female subject is determined to have an elevated plasma or serum CA-125 level (*e.g.*, greater than 25 U/mL).

[0207] In some embodiments of the disclosed method, the method is used in combination with one or more of the following diagnostic assays:

- a) the subject's annual physical examination (*e.g.*, including a HPV, and/or Pap smear screening for cervical cancer and a mammogram screening for breast cancer).
- b) plasma or serum CA-125 and/or TVUS screening test;
- c) a genetic assay to screen blood plasma for genetic mutations in circulating tumor DNA and/or protein biomarkers linked to cancer;
- d) an assay involving immunofluorescent staining to identify cell phenotype and marker expression, followed by amplification and analysis by next-generation sequencing; and
- e) BRCA1 and/or BRCA2 germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, OVA1 and OVERA tests, and/or circulating tumor cells.

[0208] In some embodiments of the disclosed method, the ovarian cancer is high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer.

[0209] In some embodiments of the disclosed method, the ovarian cancer is high-grade serous ovarian cancer. In some embodiments, the high-grade serous ovarian cancer is at an early stage.

[0210] One aspect of the disclosure herein is a method for differentiating benign adnexal mass from ovarian cancer, wherein the method comprises:

- a) detecting, in a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) from a female subject determined to have an adnexal mass, on surfaces of intact nanoparticles co-localization of at least one

biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:

- i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; and
- ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
- iii) combinations thereof;
- b) comparing the detected co-localization level with a reference level; and
- c) identifying the adnexal mass of the female subject to be likely benign when the detected co-localization level is or comparable to the reference level; or identifying the adnexal mass to be cancerous when the detected co-localization level is above the reference level.

[0211] In some embodiments, the method for differentiating benign adnexal mass from ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95%. In some embodiments, the female subject is determined to have an elevated serum CA-125 level (*e.g.*, greater than 25 U/mL).

[0212] One aspect of the disclosure herein is a method for detection of early-stage ovarian cancer, wherein the method comprises:

- a) detecting, in a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) from a female subject, on surfaces of intact nanoparticles co-localization of at least one biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:
 - i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*;
 - ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - iii) combinations thereof;
- b) comparing the detected co-localization level with a reference level; and
- c) identifying the female subject to be negative for ovarian cancer when the detected co-localization level is or comparable to the reference level; or identifying the female subject as likely to have or be susceptible to ovarian cancer, when the detected co-localization level is above the reference level.

[0213] In some embodiments, the method for detection of early-stage ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 80% to 95%. In some

embodiments, the female subject is determined to have a normal plasma or serum CA-125 level (*e.g.*, less than or equal to 25 U/mL).

[0214] In some embodiments of the disclosed method, the detecting comprises detecting on surfaces of intact nanoparticles co-localization of the at least one biomarker combination, wherein the at least one biomarker combination is selected from one of the following:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- d) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- e) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- f) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- g) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0215] In some embodiments of the disclosed method, the detecting comprises detecting on surfaces of intact nanoparticles co-localization of each of the following biomarker combinations:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen;
- d) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- e) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- f) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- g) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0216] In some embodiments of the disclosed method, the detecting comprises:

- a) capturing the intact nanoparticles from the biological sample with a capture probe that selectively interacts with the at least one capture biomarker on the intact nanoparticles;

- b) contacting the captured nanoparticles with at least one set of at least two detection probes that each selectively interacts with the at least one detection biomarker on the intact nanoparticles; and
- c) detecting a product formed when the at least two detection probes of the set are in sufficiently close proximity on the individual nanoparticles.

[0217] In some embodiments, the capture probe comprises a target-capture moiety that binds to the capture biomarker. In some embodiments, the target-capture moiety is or comprises an antibody agent directed to the capture biomarker.

[0218] In some embodiments of the disclosed method, the capture biomarker is or comprises a sialyl Lewis A antigen (also known as CA19-9), a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *MUC16*, or a sialyl Tn (sTn) antigen.

[0219] In some embodiments of the disclosed method, the capture probe is or comprises a solid substrate the disclosed method, the solid substrate comprises a magnetic bead.

[0220] In some embodiments of the disclosed method, the at least two detection probes each comprise:

- a) a target binding moiety directed to one of the at least detection biomarker; and
 - b) 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle.

[0221] In some embodiments of the disclosed method, the product was formed when the at least two detection probes of the set are in sufficiently close proximity on the individual nanoparticles such that the single-stranded overhang portions of the at least two detection probes of the set hybridize to each other to form a double-stranded complex. In some embodiments, the product formed comprises a ligated template upon contacting the double-stranded complex with a nucleic acid ligase.

[0222] In some embodiments of the disclosed method, the target binding moieties of the at least two detection probes are each directed to the same detection biomarker. In some embodiments of, the oligonucleotide domain of the at least two detection probes are different. In some embodiments, the same detection biomarker is or comprises a polypeptide encoded by human gene *BST2*. In some embodiments, the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the same detection biomarker is or comprises a sialyl Tn (sTn) antigen. In some embodiments, the target-capture moiety of the

capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.

[0223] In some embodiments of the disclosed method, the target binding moieties of the at least two detection probes are each directed to a distinct detection biomarker. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*. In some embodiments, the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*. In some embodiments, the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.

[0224] One aspect of the disclosure here is a kit comprising:

- a) at least one set of probes for a biomarker combination specific for detection of ovarian cancer,
 - wherein the biomarker combination comprises at least one capture biomarker on exosomes and at least one detection biomarker on exosomes, and wherein the capture biomarker and the detection biomarker are each independently selected from:
 - i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*;
 - ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - iii) combinations thereof; and
 - wherein the at least one set of probes comprises:
 - b) a capture probe comprising a target-capture moiety directed to the capture biomarker; and
 - c) at least two detection probes each comprising a target binding moiety directed to the at least one detection biomarker.

[0225] In some embodiments, the disclosed kit further comprises a plurality of sets of probes, each set for a distinct biomarker combination specific for detection of ovarian cancer. In some embodiments, the biomarker combination(s) is/are selected from one of the following:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- d) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- e) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- f) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- g) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0226] In some embodiments, the kit comprises at least 7 sets of probes, each set for a distinct biomarker combination as follows:

- a) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- b) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- c) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- d) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- e) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- f) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- g) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0227] In some embodiments of the disclosed kit, the capture probe and detection probes selectively bind to respective biomarkers on the exosomes with a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95%.

[0228] In some embodiments of the disclosed kit, the detection probes each further comprises 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 at least

two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same exosome.

[0229] In some embodiments of the disclosed kit, the target binding moieties of the at least two detection probes are each directed to the same detection biomarker on the exosomes. In some embodiments, the oligonucleotide domain of the at least two detection probes are different. In some embodiments, the same detection biomarker is or comprises a polypeptide encoded by human gene *BST2*. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the same detection biomarker is or comprises a sialyl Tn (sTn) antigen. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.

[0230] In some embodiments of the disclosed kit, the target binding moieties of the at least two detection probes are each directed to a distinct detection biomarker on the exosomes. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*. In some embodiments, the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*. In some embodiments, the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.

[0231] In some embodiments, the disclosed kit further comprises at least one additional reagent (*e.g.*, a ligase, a fixation agent, and/or a permeabilization agent).

[0232] These, and other aspects encompassed by the present disclosure, are described in more detail below and in the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0233] *Fig. 1* is a schematic diagram illustrating an exemplary workflow of profiling individual nanoparticles (EVs). The figure shows purification of EVs from plasma using size

exclusion chromatography (SEC) and immunoaffinity capture of EVs displaying a specific surface biomarker (*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*).

[0234] *Fig. 2* is a schematic diagram illustrating a target entity detection assay according to some embodiments described herein. In some embodiments, a target entity detection assay uses a combination of detection probes, which combination is specific for detection of cancer. In some embodiments, a duplex system includes 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). 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) 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 (*e.g.*, ovarian cancer) 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.

[0235] *Fig. 3* is a schematic diagram illustrating a target entity detection assay according to some embodiments described herein. The figure shows an exemplary triplex target entity detection system, in which in some embodiments, three or more detection probes, each for a target biomarker, can be added to a sample comprising a biological entity (*e.g.*, extracellular vesicle). In some embodiments, detection probes each comprise a target binding moiety (*e.g.*, an affinity agent such as, *e.g.*, an antibody agent against a target biomarker) 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 the corresponding single-stranded overhangs of all three or more detection probes hybridize to each other to form a linear double-stranded complex, and ligation of at least one strand of the double-stranded complex occurs, thus allowing a resulting ligated product to be detected.

[0236] *Fig. 4* is a non-limiting example of a double-stranded complex comprising four detection probes connected to each other in a linear arrangement through hybridization of their respective single-stranded overhangs.

[0237] *Fig. 5* is a schematic diagram illustrating a target entity detection assay of an exemplary embodiment described herein. In some embodiments, a plurality of detection probes, each for a distinct target, are added to a sample comprising a biological entity (*e.g.*, extracellular vesicle). In some embodiments, detection probes each comprise a target binding moiety (*e.g.*, an antibody agent) 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 all 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 form a linear double-stranded complex, and ligation of at least one strand of the resulting linear double-stranded complex occurs, thereby allowing a ligated product to be detected.

[0238] *Fig. 6* is a pie chart showing ovarian cancer prevalence by major ovarian carcinoma subtypes. “Others” refers to mixed or transitional carcinomas where it is not possible to categorize to a single subtype. See, *e.g.*, Gilks *et al.*, 2008, Seidman *et al.*, 2003, 2004, which are each incorporated herein in their entirety by reference for the purpose described herein and for additional information.

[0239] *Fig. 7* is a table that depicts delta Ct values of certain exemplary biomarker combinations that are useful for distinguishing ovarian cancer patients from control subjects (*e.g.*, healthy woman subjects and/or subjects with benign gynecological tumors and/or inflammatory conditions including, *e.g.*, Crohn’s disease, ulcerative colitis, endometriosis, *etc.*) using an exemplary assay as described herein.

[0240] *Fig. 8* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. The lower dotted red line indicates the Ct value for the healthy sample with the strongest signal and the upper dotted red line indicates the Ct value for the 10th percentile of the healthy controls. In some embodiments, benign ovarian tumor samples may be less of a concern for off-target signals than healthy control subjects and/or subjects with inflammatory conditions (*e.g.*, Crohn’s disease, ulcerative colitis, endometriosis, *etc.*). Accordingly, in some such embodiments, benign ovarian tumor samples may not be included to determine a cutoff value.

[0241] *Fig. 9* is a table that depicts delta Ct values of certain exemplary biomarker combinations that are useful for distinguishing ovarian cancer patients from control subjects (*e.g.*, healthy woman subjects and/or subjects with benign gynecological tumors and/or inflammatory conditions including, *e.g.*, Crohn’s disease, ulcerative colitis, endometriosis, *etc.*) using an exemplary assay as described herein. In some embodiments, certain biomarker combinations were selected and

ranked by the overall average delta Ct across 3 pools of subject samples from late stage HGSOc cancer patients.

[0242] *Fig. 10* is a table that depicts delta Ct values of certain exemplary biomarker combinations that are useful for distinguishing ovarian cancer patients from control subjects (*e.g.*, healthy woman subjects and/or subjects with benign gynecological tumors and/or inflammatory conditions including, *e.g.*, Crohn's disease, ulcerative colitis, endometriosis, *etc.*) using an exemplary assay as described herein. In some embodiments, certain biomarker combinations were selected and ranked by the average delta Ct of late stage HGSOc cancer patients with low CA-125 plasma levels.

[0243] *Figs. 11-15* depict performance of an exemplary assay described herein involving certain exemplary biomarker combinations. The lower dotted red line indicates the Ct value for the healthy sample with the strongest signal and the upper dotted red line indicates the Ct value for the 10th percentile of the healthy controls. In some embodiments, benign ovarian tumor samples may be less of a concern for off-target signals than healthy control subjects and/or subjects with inflammatory conditions (*e.g.*, Crohn's disease, ulcerative colitis, endometriosis, *etc.*). Accordingly, in some such embodiments, benign ovarian tumor samples may not be included to determine a cutoff value.

[0244] *Figs. 16-18* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. From left to right for each plot are shown Ct values for the "No EV" negative control, the healthy controls Pool I, the healthy controls Pool II, benign samples, early ovarian cancer, late ovarian cancer, and the positive cell line on the right.

[0245] *Fig. 19* depicts an exemplary Receiver Operating Characteristic (ROC) Curve for distinguishing patients with early stage I-II ovarian cancer from healthy/benign ovarian mass patients. Curves were generated using the Ct values determined from the exemplary assays shown in *Fig. 1*. Curves depict an area under the curve (AUC) of 0.94 when utilizing sTn antigen, BST2 + MUC1 biomarker combination and an AUC of 0.85 when utilizing plasma CA-125 levels.

[0246] *Fig. 20* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. (A-D) Box and whisker plots for certain exemplary biomarker combinations. From left to right for each plot are shown Ct values for "No EV" negative control, healthy controls, benign samples, early-stage ovarian cancer, late-stage ovarian cancer, and cell line positive control. (E-H) Corresponding Receiver Operating Characteristic (ROC) Curves for distinguishing patients with ovarian cancer (including early- and late-stage ovarian cancer patients) from both healthy and benign ovarian mass patients.

[0247] *Fig. 21* depicts exemplary Receiver Operating Characteristic (ROC) curves for distinguishing patients with ovarian cancer from healthy patients (A) or from both healthy and benign ovarian mass patients (B).

[0248] *Fig. 22* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. **(A-G)** For each plot are shown Ct values (subtracted from 40) for healthy controls, benign samples, early-stage ovarian cancer, and late stage ovarian cancer.

[0249] *Fig. 23* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. **(A-C)** For each plot are shown Ct values (subtracted from 40) for healthy controls, benign samples, early-stage ovarian cancer, and late stage ovarian cancer.

[0250] *Fig. 24* depicts performance of plasma CA-125 for distinguishing early stage and late stage ovarian cancer from both healthy and benign samples as measured by ELISA. From left to right is shown U/mL (log2) for healthy controls, benign samples, early-stage ovarian cancer, and late stage ovarian cancer.

[0251] *Fig. 25 (A-D)* depicts performance of an exemplary assay described herein involving certain exemplary biomarker combinations. From left to right for each plot are shown Ct values for healthy controls, early-stage ovarian cancer, late-stage ovarian cancer, adenofibroma, fibroma, other, cyst, cystadenoma, no evidence of malignancy, endometriosis, leiomyoma, teratoma, and cystadenofibroma.

[0252] *Fig. 26* depicts performance of **(A)** an exemplary assay described herein involving an indicated biomarker combination and **(B)** CA-125 level for differentiating ovarian cancer from exemplary off-target cancers. From left to right for each plot are shown Ct values for healthy controls, benign samples, early-stage ovarian cancer, late-stage ovarian cancer, uterine cancer, lung cancer, pancreatic cancer, colorectal cancer (CRC), breast cancer, and bladder cancer.

[0253] *Fig. 27* depicts performance of **(A)** an exemplary assay described herein involving a specific biomarker combination and **(B)** CA-125 level for differentiating ovarian cancer from exemplary inflammatory conditions. From left to right for each plot are shown Ct values for healthy controls, benign samples, early-stage ovarian cancer, late-stage ovarian cancer, Crohn's disease, Type 2 Diabetes, endometriosis, acute pancreatitis, rheumatoid arthritis, and ulcerative colitis.

[0254] *Fig. 28* depicts exemplary Receiver Operating Characteristics (ROC) Curves to show performance of an exemplary assay described herein involving a specific set of biomarker combination as shown in **Table 8**, relative to CA-125 test. **(A)** ROC curves for differentiating patients with ovarian cancer (including both early-stage and late-stage patients) from both healthy and benign ovarian mass patients. The McNemar p-value comparing the CA-125 curve and the set of 7 biomarker combinations curve at 99% specificity was <0.0001 (p-value: 4.71e-12). **(B)** ROC curves for differentiating patients with early-stage ovarian cancer (*e.g.*, stage I and/or stage II HGSOE cases) from both healthy and benign ovarian mass patients. The McNemar p-value

comparing the CA-125 curve and the set of 7 biomarker combinations curve at 99% specificity was <0.0001 (p-value: $2.99\text{e-}07$).

[0255] *Fig. 29* depicts exemplary Receiver Operating Characteristics (ROC) curves to show performance of an exemplary assay described herein involving a specific set of biomarker combination as shown in **Table 8**, relative to CA-125 test. **(A)** ROC curves for differentiating patients with early-stage ovarian cancer (*e.g.*, stage I and stage II HGSOc cases) from healthy patients. The McNemar p-value comparing the CA-125 ROC curve and the set of 7 biomarker combinations ROC curve at 99% specificity was 1. **(B)** ROC curves for differentiating patients with early-stage ovarian cancer (*e.g.*, stage I and stage II HGSOc cases) from benign ovarian mass patients. The McNemar p-value comparing the CA-125 ROC curve and the set of 7 biomarker combinations ROC curve at 99% specificity was <0.0001 .

CERTAIN DEFINITIONS

[0256] *Administering*: As used herein, the term “administering” or “administration” typically refers to the administration of a composition to a subject to achieve delivery of an agent that is, or is included in, a composition to a target site or a site to be treated. Those of ordinary skill in the art will be aware of a variety of routes that may, in appropriate circumstances, be utilized for administration to a subject, for example a human. For example, in some embodiments, administration may be parenteral. In some embodiments, administration may be oral. In some embodiments, administration may involve only a single dose. In some embodiments, administration may involve application of a fixed number of doses. In some embodiments, administration may involve dosing that is intermittent (*e.g.*, a plurality of doses separated in time) and/or periodic (*e.g.*, individual doses separated by a common period of time) dosing. In some embodiments, administration may involve continuous dosing (*e.g.*, perfusion) for at least a selected period of time.

[0257] *Adnexal mass*: As used herein, the term “adnexal mass” refers to a growth or lump in tissue around the uterus. In some embodiments, an adnexal mass may develop in one or more ovaries. In some embodiments, an adnexal mass may develop in one or more fallopian tubes. In some embodiments, an adnexal mass may develop in neighboring connective tissues around the uterus. In some embodiments, an adnexal mass may be a benign tumor. Examples of a benign adnexal mass include, but are not limited to adenofibroma, fibroma, ovarian cyst, cystadenoma, endometriosis, leiomyoma, teratoma, cystadenofibroma, *etc.* In some embodiments, an adnexal mass may be a malignant tumor.

[0258] *Affinity Agent*: The term “affinity agent” as used herein refers to an entity that is or comprises a target-binding moiety as described herein, and therefore binds to a target of interest (*e.g.*, molecular target of interest such as a biomarker or an epitope). In many embodiments, an affinity agent in accordance with the present disclosure binds specifically with a biomarker as

described herein. In many embodiments, an affinity agent in accordance with the present disclosure binds specifically with a surface biomarker as described herein. In some embodiments, an affinity agent in accordance with the present disclosure binds specifically with a carbohydrate-dependent marker as described herein. In some embodiments, an affinity agent may be or comprise an antibody agent (*e.g.*, an antibody or other entity that is or includes an antigen-binding portion thereof). Alternatively, or additionally, in some embodiments, an affinity agent may be selected from the group consisting of affimers, aptamers, lectins, sialic acid-binding immunoglobulin-type lectins (siglecs), and combinations thereof, and/or another binding agent that may be considered a ligand. In some embodiments, a target (*e.g.*, a biomarker target) of an affinity agent is or comprises one or more polypeptide, nucleic acid, carbohydrate, and/or lipid moieties and/or entities).

[0259] *Agent:* In general, the term “agent”, as used herein, is used to refer to an entity (*e.g.*, for example, a lipid, metal, nucleic acid, polypeptide, polysaccharide, small molecule, etc., or complex, combination, mixture or system [*e.g.*, cell, tissue, organism] thereof), or phenomenon (*e.g.*, heat, electric current or field, magnetic force or field, etc). In appropriate circumstances, as will be clear from context to those skilled in the art, the term may be utilized to refer to an entity that is or comprises a cell or organism, or a fraction, extract, or component thereof. Alternatively, or additionally, as context will make clear, the term may be used to refer to a natural product in that it is found in and/or is obtained from nature. In some instances, again as will be clear from context, the term may be used to refer to one or more entities that is man-made in that it is designed, engineered, and/or produced through action of the hand of man and/or is not found in nature. In some embodiments, an agent may be utilized in isolated or pure form; in some embodiments, an agent may be utilized in crude form. In some embodiments, potential agents may be provided as collections or libraries, for example that may be screened to identify or characterize active agents within them. In some cases, the term “agent” may refer to a compound or entity that is or comprises a polymer; in some cases, the term may refer to a compound or entity that comprises one or more polymeric moieties. In some embodiments, the term “agent” may refer to a compound or entity that is not a polymer and/or is substantially free of any polymer and/or of one or more particular polymeric moieties. In some embodiments, the term may refer to a compound or entity that lacks or is substantially free of any polymeric moiety.

[0260] *Amplification:* The terms “amplification” and “amplify” refers to a template-dependent process that results in an increase in the amount and/or levels of a nucleic acid molecule relative to its initial amount and/or level. A template-dependent process is generally a process that involves template-dependent extension of a primer molecule, wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson, J. D. *et al.*, *In: Molecular Biology of the Gene*, 4th Ed., W. A. Benjamin,

Inc., Menlo Park, Calif. (1987); which is incorporated herein by reference for the purpose described herein).

[0261] *Antibody agent:* As used herein, the term “antibody agent” refers to an agent that specifically binds to a particular antigen. In some embodiments, the term encompasses any polypeptide or polypeptide complex that includes immunoglobulin structural elements sufficient to confer specific binding. Exemplary antibody agents include but are not limited to monoclonal antibodies or polyclonal antibodies. In some embodiments, an antibody agent may include one or more constant region sequences that are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, an antibody agent may include one or more sequence elements are humanized, primatized, chimeric, *etc.*, as is known in the art. In many embodiments, the term “antibody agent” is used to refer to one or more of the art-known or developed constructs or formats for utilizing antibody structural and functional features in alternative presentation. For example, embodiments, an antibody agent utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE or IgM antibodies; bi- or multi- specific antibodies (*e.g.*, Zybodies®, *etc.*); antibody fragments such as Fab fragments, Fab’ fragments, F(ab’)2 fragments, Fd’ fragments, Fd fragments, and isolated complementary determining regions (CDRs) or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (*e.g.*, shark single domain antibodies such as IgNAR or fragments thereof); camelid antibodies; masked antibodies (*e.g.*, Probodies®); Small Modular ImmunoPharmaceuticals (“SMIPs™”); single chain or Tandem diabodies (TandAb®); VHs; Anticalins®; Nanobodies® minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies; Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®, KALBITOR®s, and Affimers®. In some embodiments, an antibody may lack a covalent modification (*e.g.*, attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody may contain a covalent modification (*e.g.*, attachment of a glycan, a payload [*e.g.*, a detectable moiety, a therapeutic moiety, a catalytic moiety, *etc.*], or other pendant group [*e.g.*, poly-ethylene glycol, *etc.*]). In many embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes one or more structural elements recognized by those skilled in the art as a complementarity determining region (CDR); in some embodiments an antibody agent is or comprises a polypeptide whose amino acid sequence includes at least one CDR (*e.g.*, at least one heavy chain CDR and/or at least one light chain CDR) that is substantially identical to one found in a reference antibody. In some embodiments an included CDR is substantially identical to a reference CDR in that it is either identical in sequence or contains between 1-5 amino acid substitutions as compared with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some

embodiments an included CDR is substantially identical to a reference CDR in that it shows at least 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that at least one amino acid within the included CDR is substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical with that of the reference CDR. In some embodiments an included CDR is substantially identical to a reference CDR in that 1-5 amino acids within the included CDR are deleted, added, or substituted as compared with the reference CDR but the included CDR has an amino acid sequence that is otherwise identical to the reference CDR. In some embodiments, an antibody agent is or comprises a polypeptide whose amino acid sequence includes structural elements recognized by those skilled in the art as an immunoglobulin variable domain. In some embodiments, an antibody agent is a polypeptide protein having a binding domain which is homologous or largely homologous to an immunoglobulin-binding domain.

[0262] Antibody agents can be made by the skilled person using methods and commercially available services and kits known in the art. For example, methods of preparation of monoclonal antibodies are well known in the art and include hybridoma technology and phage display technology. Further antibodies suitable for use in the present disclosure are described, for example, in the following publications: *Antibodies A Laboratory Manual*, Second edition. Edward A. Greenfield. Cold Spring Harbor Laboratory Press (September 30, 2013); *Making and Using Antibodies: A Practical Handbook*, Second Edition. Eds. Gary C. Howard and Matthew R. Kaser. CRC Press (July 29, 2013); *Antibody Engineering: Methods and Protocols*, Second Edition (Methods in Molecular Biology). Patrick Chames. Humana Press (August 21, 2012); *Monoclonal Antibodies: Methods and Protocols* (Methods in Molecular Biology). Eds. Vincent Ossipow and Nicolas Fischer. Humana Press (February 12, 2014); and *Human Monoclonal Antibodies: Methods and Protocols* (Methods in Molecular Biology). Michael Steinitz. Humana Press (September 30, 2013)).

[0263] Antibodies may be produced by standard techniques, for example by immunization with the appropriate polypeptide or portion(s) thereof, or by using a phage display library. If polyclonal antibodies are desired, a selected host animal (*e.g.*, mouse, rabbit, goat, horse, chicken, *etc.*) is immunized with an immunogenic polypeptide bearing a desired epitope(s), optionally

happenized to another polypeptide. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface-active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to the desired epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography or any other method known in the art. Techniques for producing and processing polyclonal antisera are well known in the art.

[0264] *Antigen*: As used herein, the term “antigen” refers to an entity (e.g., a molecule or a molecular structure such as, e.g., a peptide or protein, carbohydrate, lipoparticle, oligonucleotide, chemical molecule, or combinations thereof) that includes one or more epitopes and therefore is recognized and bound by an affinity agent (e.g., an antibody, affimer, or aptamer).

[0265] *Approximately or about*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In general, those skilled in the art, familiar within the context, will appreciate the relevant degree of variance encompassed by “about” or “approximately” in that context. For example, in some embodiments, the term “approximately” or “about” may encompass a range of values that are within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less of the referred value.

[0266] *Aptamer*: As used herein, the term “aptamer” typically refers to a nucleic acid molecule or a peptide molecule that binds to a specific target molecule (e.g., an epitope). In some embodiments, a nucleic acid aptamer may be described by a nucleotide sequence and is typically about 15-60 nucleotides in length. A nucleic acid aptamer may be or comprise a single stranded and/or double-stranded structure. In some embodiments, a nucleic acid aptamer may be or comprise DNA. In some embodiments, a nucleic acid aptamer may be or comprise RNA. Without wishing to be bound by any theory, it is contemplated that the chain of nucleotides in an aptamer form intramolecular interactions that fold the molecule into a complex three-dimensional shape, and this three-dimensional shape allows the aptamer to bind tightly to the surface of its target molecule. In some embodiments, a peptide aptamer may be described to have one or more peptide loops of variable sequence displayed by a protein scaffold. Peptide aptamers can be isolated from combinatorial libraries and often subsequently improved by directed mutation or rounds of variable region mutagenesis and selection. Given the extraordinary diversity of molecular shapes that exist within the universe of all possible nucleotide and/or peptide sequences, aptamers may be obtained for a wide array of molecular targets, including proteins and small molecules. In addition to high specificity, aptamers typically have very high affinities for their targets (e.g., affinities in the

picomolar to low nanomolar range for proteins or polypeptides). Because aptamers are typically synthetic molecules, aptamers are amenable to a variety of modifications, which can optimize their function for particular applications.

[0267] *Associated with:* Two events or entities are “associated” with one another, as that term is used herein, if the presence, level and/or form of one is correlated with that of the other. For example, a particular biological phenomenon (*e.g.*, expression of a specific biomarker) is considered to be associated with ovarian cancer (*e.g.*, a specific type of ovarian cancer and/or stage of ovarian cancer), if its presence correlates with incidence of and/or susceptibility of the ovarian cancer (*e.g.*, across a relevant population).

[0268] *Biological entity:* In appropriate circumstances, as will be clear from context to those skilled in the art, the term “biological entity” may be utilized to refer to an entity or component that is present in a biological sample, *e.g.*, in some embodiments derived or obtained from a subject, which, in some embodiments, may be or comprise a cell or an organism, such as an animal or human, or, in some embodiments, may be or comprise a biological tissue or fluid. In some embodiments, a biological entity is or comprises a cell or microorganism, or a fraction, extract, or component thereof (including, *e.g.*, intracellular components and/or molecules secreted by a cell or microorganism). For example, in some embodiments, a biological entity is or comprises a cell. In some embodiments, a biological entity is or comprises an extracellular vesicle. In some embodiments, a biological entity is or comprises a biological analyte (*e.g.*, a metabolite, carbohydrate, protein or polypeptide, enzyme, lipid, organelle, cytokine, receptor, ligand, and any combinations thereof). In some embodiments, a biological entity present in a sample is in a native state (*e.g.*, proteins or polypeptides remain in a naturally occurring conformational structure). In some embodiments, a biological entity is processed, *e.g.*, by isolating from a sample or deriving from a naturally occurring biological entity. For example, a biological entity can be processed with one or more chemical agents such that it is more desirable for detection utilizing technologies provided herein. In some embodiments, a biological entity is or comprises a nanoparticle having a size within the range of about 30 nm to about 1000 nm, which in some embodiments are obtained from a bodily fluid sample (*e.g.*, but not limited to a blood sample) of a subject. In some embodiments, such a nanoparticle may be or comprise a protein aggregate, including, *e.g.*, in some embodiments comprising a glycan, and/or an extracellular vesicle. In some embodiments, such a nanoparticle may have a size within the range of about 30 nm to about 1000 nm, about 50 nm to about 500 nm, or about 75 nm to about 500 nm. As an example only, a biological entity may be a cell or extracellular vesicle that is contacted with a fixative agent (*e.g.*, but not limited to methanol and/or formaldehyde) to cause proteins and/or peptides present in the cell or extracellular vesicle to form crosslinks. In some embodiments, a biological entity is in an isolated or pure form (*e.g.*, isolated from a bodily fluid sample such as, *e.g.*, a blood, serum, plasma sample, *etc.*). In some embodiments, a biological

entity may be present in a complex matrix (*e.g.*, a bodily fluid sample such as, *e.g.*, a blood, serum, or plasma sample, *etc.*).

[0269] *Biomarker:* The term “biomarker” typically refers to an entity, event, or characteristic whose presence, level, degree, type, and/or form, correlates with a particular biological event or state of interest, so that it is considered to be a “marker” of that event or state. To give but a few examples, in some embodiments, a biomarker may be or comprise a marker for a particular disease state, or for likelihood that a particular disease, disorder or condition may develop, occur, or reoccur. In some embodiments, a biomarker may be or comprise a marker for a particular disease or therapeutic outcome, or likelihood thereof. In some embodiments, a biomarker may be or comprise a marker for a particular tissue (*e.g.*, but not limited to brain, breast, colon, ovary and/or other tissues associated with a female reproductive system, pancreas, prostate and/or other tissues associated with a male reproductive system, liver, lung, and skin). Such a marker for a particular tissue, in some embodiments, may be specific for a healthy tissue, specific for a diseased tissue, or in some embodiments may be present in a normal healthy tissue and diseased tissue (*e.g.*, a tumor); those skilled in the art, reading the present disclosure, will appreciate appropriate contexts for each such type of biomarker. In some embodiments, a biomarker may be or comprise a cancer-specific marker (*e.g.*, a marker that is specific to a particular cancer). In some embodiments, a biomarker may be or comprise a non-specific cancer marker (*e.g.*, a marker that is present in at least two or more cancers). A non-specific cancer marker may be or comprise, in some embodiments, a generic marker for cancers (*e.g.*, a marker that is typically present in cancers, regardless of tissue types), or in some embodiments, a marker for cancers of a specific tissue (*e.g.*, but not limited to brain, breast, colon, ovary and/or other tissues associated with a female reproductive system, pancreas, prostate and/or other tissues associated with a male reproductive system, liver, lung, and skin). Thus, in some embodiments, a biomarker is predictive; in some embodiments, a biomarker is prognostic; in some embodiments, a biomarker is diagnostic of the relevant biological event or state of interest. A biomarker may be or comprise an entity of any chemical class and may be or comprise a combination of entities. For example, in some embodiments, a biomarker may be or comprise a nucleic acid, a polypeptide, a lipid, a carbohydrate, a small molecule, an inorganic agent (*e.g.*, a metal or ion), or a combination thereof. In some embodiments, a biomarker is or comprises a portion of a particular molecule, complex, or structure; *e.g.*, in some embodiments, a biomarker may be or comprise an epitope. In some embodiments, a biomarker is a surface marker (*e.g.*, a surface protein marker) of an extracellular vesicle associated with ovarian cancer. In some embodiments, a biomarker is intravesicular (*e.g.*, a protein or RNA marker that is present within an extracellular vesicle). In some embodiments, a biomarker may be or comprise a genetic or epigenetic signature. In some embodiments, a biomarker may be or comprise a gene expression signature. In some embodiments, a “biomarker” appropriate for use in accordance with the present disclosure may refer

to presence, level, and/or form of a molecular entity (*e.g.*, epitope) present in a target marker. For example, in some embodiments, two or more “biomarkers” as molecular entities (*e.g.*, epitopes) may be present on the same target marker (*e.g.*, a marker protein such as a surface protein present in an extracellular vesicle).

[0270] *Blood-derived sample:* The term “blood-derived sample,” as used herein, refers to a sample derived from a blood sample (*i.e.*, a whole blood sample) of a subject in need thereof. Examples of blood-derived samples include, but are not limited to, blood plasma (including, *e.g.*, fresh frozen plasma), blood serum, blood fractions, plasma fractions, serum fractions, blood fractions comprising red blood cells (RBC), platelets, leukocytes, *etc.*, and cell lysates including fractions thereof (for example, cells, such as red blood cells, white blood cells, *etc.*, may be harvested and lysed to obtain a cell lysate). In some embodiments, a blood-derived sample that is used with methods, systems, and/or kits described herein is a plasma sample.

[0271] *Cancer:* The term “cancer” is used herein to generally refer to a disease or condition in which cells of a tissue of interest exhibit relatively abnormal, uncontrolled, and/or autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. In some embodiments, cancer may comprise cells that are precancerous (*e.g.*, benign), malignant, pre-metastatic, metastatic, and/or non-metastatic. The present disclosure provides technologies for detection of ovarian cancer.

[0272] *Capture assay:* As used herein, the term “capture assay” refers to a process of isolating or separating a biological entity of interest from a sample (*e.g.*, in some embodiments a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample)). In some embodiments, a biological entity of interest is isolated or separated from a sample (*e.g.*, in some embodiments a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample)) using a capture probe described herein. In some embodiments, a biological entity of interest that binds to a capture probe described herein is subject to a detection assay described herein. In some embodiments, a biological entity of interest amenable to a capture assay described herein is or comprises nanoparticles having a size range of interest that includes extracellular vesicles. In some embodiments, such a nanoparticle may have a size within the range of about 30 nm to about 1000 nm, about 50 nm to about 500 nm, or about 75 nm to about 500 nm. In some embodiments, a biological entity of interest amenable to a capture assay described herein is or comprises extracellular vesicles (*e.g.*, in some embodiments exosomes) of interest.

[0273] *Capture probe:* As used herein, the term “capture probe” refers to a capture agent for capturing a biological entity of interest from a sample (*e.g.*, in some embodiments a bodily fluid-derived sample, *e.g.*, but not limited to a blood-derived sample). In many embodiments described herein, a capture agent comprises at least one target-capture moiety that binds to a surface

polypeptide of a biological entity of interest. In some embodiments, such a biological entity of interest is or comprises nanoparticles having a size range of interest that includes extracellular vesicles. In some embodiments, such nanoparticles may have a size within the range of about 30 nm to about 1000 nm, about 50 nm to about 500 nm, or about 75 nm to about 500 nm. In some embodiments, such a biological entity of interest comprises extracellular vesicles (e.g., in some embodiments exosomes). In some embodiments, a capture agent comprises at least one target moiety that binds to a surface biomarker (e.g., ones described herein) of nanoparticles having a size within the range of about 30 nm to about 1000 nm, including, e.g., extracellular vesicles (e.g., in some embodiments exosomes). In some embodiments, a target-capture moiety of a capture agent is or comprises an affinity agent described herein. In some embodiments, a target-capture moiety of a capture agent is or comprises an antibody agent. In some embodiments, a target-capture moiety of a capture agent is or comprises a lectin or a sialic acid-binding immunoglobulin-type lectin. In some embodiments, a capture agent may comprise a solid substrate such that its target-capture moiety is immobilized thereonto. In some embodiments, an exemplary solid substrate is a bead (e.g., a magnetic bead). In some embodiments, a capture probe is or comprises a population of magnetic beads comprising a target-capture moiety that specifically binds to a surface biomarker described herein.

[0274] *Classification cutoff:* As used herein, the term “classification cutoff” refers to a level, value, or score, or a set of values, or an indicator that is used to predict a subject’s risk for a disease or condition (e.g., ovarian cancer), for example, by defining one or more dividing lines among two or more subsets of a population (e.g., normal healthy subjects and subjects with inflammatory conditions vs. ovarian cancer subjects). In some embodiments, a classification cutoff may be determined referencing at least one reference threshold level (e.g., reference cutoff) for a target biomarker signature described herein, optionally in combination with other appropriate variables, e.g., age, life-history-associated risk factors, hereditary factors, physical and/or medical conditions of a subject. In some embodiments where a classification is based on a single target biomarker signature (e.g., as described herein), a classification cutoff may be the same as a reference threshold (e.g., cutoff) pre-determined for the single target biomarker signature. In some embodiments where a classification is based on two or more target biomarker signatures, a classification cutoff may reference two or more reference thresholds (e.g., cutoffs) each individually pre-determined for the corresponding target biomarker signatures, and optionally incorporate one or more appropriate variables, e.g., age, life-history-associated risk factors, hereditary factors, physical and/or medical conditions of a subject. In some embodiments, a classification cutoff may be determined via a computer algorithm-mediated analysis that references at least one reference threshold level (e.g., reference cutoff) for a target biomarker signature described herein, optionally in

combination with other appropriate variables, *e.g.*, age, life-history-associated risk factors, hereditary factors, physical and/or medical conditions of a subject.

[0275] *Close proximity*: The term “close proximity” as used herein, refers to a distance between two detection probes (*e.g.*, two detection probes in a pair) that is sufficiently close enough such that an interaction between the detection probes (*e.g.*, through respective oligonucleotide domains) is expected to likely occur. For example, in some embodiments, probability of two detection probes interacting with each other (*e.g.*, through respective oligonucleotide domains) over a period of time when they are in sufficiently close proximity to each other under a specified condition (*e.g.*, when detection probes are bound to respective targets in an extracellular vesicle is at least 50% or more, including, *e.g.*, at least 60%, at least 70%, at least 80%, at least 90% or more. In some embodiments, a distance between two detection probes when they are in sufficiently close proximity to each other may range between approximately 0.1-1000 nm, or 0.5-500 nm, or 1-250 nm. In some embodiments, a distance between two detection probes when they are in sufficiently close proximity to each other may range between approximately 0.1-10 nm or between approximately 0.5-5 nm. In some embodiments, a distance between two detection probes when they are in sufficiently close proximity to each other may be less than 100 nm or shorter, including, *e.g.*, less than 90 nm, less than 80 nm, less than 70 nm, less than 60 nm, less than 50 nm, less than 40 nm, less than 30 nm, less than 20 nm, less than 10 nm, less than 5 nm, less than 1 nm, or shorter. In some embodiments, a distance between two detection probes when they are in sufficiently close proximity to each other may range between approximately 40-1000 nm or 40 nm-500 nm.

[0276] *Comparable*: As used herein, the term “comparable” refers to two or more agents, entities, situations, sets of conditions, *etc.*, that may not be identical to one another but that are sufficiently similar to permit comparison therebetween so that one skilled in the art will appreciate that conclusions may reasonably be drawn based on differences or similarities observed. In some embodiments, comparable sets of conditions, circumstances, individuals, or populations are characterized by a plurality of substantially identical features and one or a small number of varied features. Those of ordinary skill in the art will understand, in context, what degree of identity is required in any given circumstance for two or more such agents, entities, situations, sets of conditions, *etc.* to be considered comparable. For example, those of ordinary skill in the art will appreciate that sets of circumstances, individuals, or populations are comparable to one another when characterized by a sufficient number and type of substantially identical features to warrant a reasonable conclusion that differences in results obtained or phenomena observed under or with different sets of circumstances, individuals, or populations are caused by or indicative of the variation in those features that are varied.

[0277] *Complementary*: As used herein, the term “complementary” is used in reference to oligonucleotide hybridization related by base-pairing rules. For example, the sequence “C-A-G-T” is

complementary to the sequence “G-T-C-A.” Complementarity can be partial or total. Thus, any degree of partial complementarity is intended to be included within the scope of the term “complementary” provided that the partial complementarity permits oligonucleotide hybridization. Partial complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. Total or complete complementarity between nucleic acids is where each and every nucleic acid base is matched with another base under the base pairing rules.

[0278] *Detecting:* The term “detecting” is used broadly herein to include appropriate means of determining the presence or absence of an extracellular vesicle expressing a target biomarker signature of ovarian cancer or any form of measurement indicative of such an extracellular vesicle. Thus, “detecting” may include determining, measuring, assessing, or assaying the presence or absence, level, amount, and/or location of an entity of interest (*e.g.*, a surface protein biomarker, an intravesicular protein biomarker, or an intravesicular RNA biomarker) that corresponds to part of a target biomarker signature in any way. In some embodiments, “detecting” may include determining, measuring, assessing, or quantifying a form of measurement indicative of an entity of interest (*e.g.*, a ligated template indicative of a surface protein biomarker and/or an intravesicular protein biomarker, or a PCR amplification product indicative of an intravesicular mRNA). Quantitative and qualitative determinations, measurements or assessments are included, including semi-quantitative. Such determinations, measurements or assessments may be relative, for example when an entity of interest (*e.g.*, a surface protein biomarker, an intravesicular protein biomarker, or an intravesicular RNA biomarker) or a form of measurement indicative thereof is being detected relative to a control reference, or absolute. As such, the term “quantifying” when used in the context of quantifying an entity of interest (*e.g.*, a surface protein biomarker, an intravesicular protein biomarker, or an intravesicular RNA biomarker) or a form of measurement indicative thereof can refer to absolute or to relative quantification. Absolute quantification may be accomplished by correlating a detected level of an entity of interest (*e.g.*, a surface protein biomarker, an intravesicular protein biomarker, or an intravesicular RNA biomarker) or a form of measurement indicative thereof to known control standards (*e.g.*, through generation of a standard curve). Alternatively, relative quantification can be accomplished by comparison of detected levels or amounts between two or more different entities of interest (*e.g.*, different surface protein biomarkers, intravesicular protein biomarkers, or intravesicular RNA biomarkers) to provide a relative quantification of each of the two or more different entities of interest, *i.e.*, relative to each other.

[0279] *Detection label:* The term “detection label” as used herein refers to any element, molecule, functional group, compound, fragment or moiety that is detectable. In some embodiments, a detection label is provided or utilized alone. In some embodiments, a detection label is provided and/or utilized in association with (*e.g.*, joined to) another agent. Examples of detection labels include, but are not limited to: various ligands, radionuclides (*e.g.*, ^3H , ^{14}C , ^{18}F , ^{19}F , ^{32}P , ^{35}S , ^{135}I , ^{125}I ,

¹²³I, ⁶⁴Cu, ¹⁸⁷Re, ¹¹¹In, ⁹⁰Y, ^{99m}Tc, ¹⁷⁷Lu, ⁸⁹Zr, *etc.*), fluorescent dyes, chemiluminescent agents (such as, for example, acridinium esters, stabilized dioxetanes, and the like), bioluminescent agents, spectrally resolvable inorganic fluorescent semiconductor nanocrystals (*i.e.*, quantum dots), metal nanoparticles (*e.g.*, gold, silver, copper, platinum, *etc.*) nanoclusters, paramagnetic metal ions, enzymes, colorimetric labels (such as, for example, dyes, colloidal gold, and the like), biotin, digoxigenin, haptens, and proteins for which antisera or monoclonal antibodies are available.

[0280] *Detection probe:* The term “detection probe” typically refers to a probe directed to detection and/or quantification of a specific target. In some embodiments, a detection probe is a quantification probe, which provides an indicator representing level of a specific target. In accordance with the present disclosure, a detection probe refers to a composition comprising a target binding entity, directly or indirectly, coupled to an oligonucleotide domain, wherein the target binding entity specifically binds to a respective target (*e.g.*, molecular target), and wherein at least a portion of the oligonucleotide domain is designed to permit hybridization with a portion of an oligonucleotide domain of another detection probe for a distinct target. In many embodiments, an oligonucleotide domain appropriate for use in the accordance with the present disclosure comprises a double-stranded portion and at least one single-stranded overhang. In some embodiments, an oligonucleotide domain may comprise a double-stranded portion and a single-stranded overhang at each end of the double-stranded portion. In some embodiments, a target binding entity of a detection probe is or comprises an affinity agent described herein. In some embodiments, a target binding entity of a detection probe is or comprises an antibody agent. In some embodiments, a target binding entity of a detection probe is or comprises a lectin or a sialic acid-binding immunoglobulin-type lectin (siglec).

[0281] *Double-stranded:* As used herein, the term “double-stranded” in the context of oligonucleotide domain is understood by those of skill in the art that a pair of oligonucleotides exist in a hydrogen-bonded, helical arrangement typically associated with, for example, nucleic acid such as DNA. In addition to the 100% complementary form of double-stranded oligonucleotides, the term “double-stranded” as used herein is also meant to refer to those forms which include mismatches (*e.g.*, partial complementarity) and/or structural features as bulges, loops, or hairpins.

[0282] *Double-stranded complex:* As used herein, the term “double-stranded complex” typically refers to a complex comprising at least two or more (including, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) detection probes (*e.g.*, as provided and/or utilized herein), each directed to a target (which can be the same target or a distinct target), connected or coupled to one another in a linear arrangement through hybridization of complementary single-stranded overhangs of the detection probes. In some embodiments, such a double-stranded complex may comprise an extracellular vesicle, wherein respective target binding moieties of the detection probes are simultaneously bound to the extracellular vesicle.

[0283] *Epitope:* As used herein, the term “epitope” includes any moiety that is specifically recognized by an immunoglobulin (*e.g.*, antibody or receptor) binding component or an aptamer. In some embodiments, an epitope is comprised of a plurality of chemical atoms or groups on an antigen. In some embodiments, such chemical atoms or groups are surface-exposed when the antigen adopts a relevant three-dimensional conformation. In some embodiments, such chemical atoms or groups are physically near to each other in space when the antigen adopts such a conformation. In some embodiments, at least some such chemical atoms or groups are physically separated from one another when the antigen adopts an alternative conformation (*e.g.*, is linearized).

[0284] *Extracellular vesicle:* As used herein, the term “extracellular vesicle” typically refers to a vesicle outside of a cell, *e.g.*, secreted by a cell. Examples of secreted vesicles include, but are not limited to exosomes, microvesicles, microparticles, ectosomes, oncosomes, and apoptotic bodies. Without wishing to be bound by theory, exosomes are nanometer-sized vesicles (*e.g.*, between 40 nm and 120 nm) of endocytic origin that may form by inward budding of the limiting membrane of multivesicular endosomes (MVEs), while microvesicles typically bud from the cell surface and their size may vary between 50 nm and 1000 nm. In some embodiments, an extracellular vesicle is or comprises an exosome and/or a microvesicle. In some embodiments, a sample comprising an extracellular vesicle is substantially free of apoptotic bodies. In some embodiments, a sample comprising nanoparticles may comprise nanoparticles shed or derived from one or more tissues (*e.g.*, cancerous tissues and/or non-cancerous or healthy tissues). In some embodiments, an extracellular vesicle in a sample may be shed or derived from an ovarian cancer tumor; in some embodiments, an extracellular vesicle is shed or derived from a tumor of a non-ovarian cancer. In some embodiments, an extracellular vesicle is shed or derived from a healthy tissue. In some embodiments, an extracellular vesicle is shed or derived from a benign gynecological tumor. In some embodiments, an extracellular vesicle is shed or derived from a tissue of a subject with symptoms (*e.g.*, non-specific symptoms) associated with ovarian cancer.

[0285] *Extracellular vesicle-associated membrane-bound polypeptide:* As used herein, such a term refers to a polypeptide that is present in the membrane of an extracellular vesicle. In some embodiments, such a polypeptide may be tumor-specific. In some embodiments, such a polypeptide may be tissue-specific (*e.g.*, ovarian tissue-specific). In some embodiments, such a polypeptide may be non-specific, *e.g.*, it is present in one or more non-target tumors, and/or in one or more non-target tissues.

[0286] *Hybridization:* As used herein, the term “hybridizing”, “hybridize”, “hybridization”, “annealing”, or “anneal” are used interchangeably in reference to pairing of complementary nucleic acids using any process by which a strand of nucleic acid joins with a complementary strand through base pairing to form a hybridization complex. Hybridization and the strength of hybridization (*e.g.*, strength of the association between the nucleic acids) is impacted by various factors including, *e.g.*,

the degree of complementarity between the nucleic acids, stringency of the conditions involved, the melting temperature (T) of the formed hybridization complex, and the G:C ratio within the nucleic acids.

[0287] *Intravesicular protein biomarker:* As used herein, the term “intravesicular protein biomarker” refers to a marker indicative of the state (*e.g.*, presence, level, and/or activity) of a polypeptide that is present within a biological entity (*e.g.*, a cell or an extracellular vesicle). In many embodiments, an intravesicular protein biomarker is associated with or present within an extracellular vesicle. In some embodiments, an intravesicular protein biomarker may be or comprise a phosphorylated polypeptide. In some embodiments, an intravesicular protein biomarker may be or comprise a mutated polypeptide. In some embodiments, non-limiting examples of intravesicular biomarkers (*e.g.*, intravesicular protein biomarkers) that are useful for ovarian cancer detection include CRABP2, KLK7, MIF, PRAME, S100A1, or combinations thereof.

[0288] *Intravesicular RNA biomarker:* As used herein, the term “intravesicular RNA biomarker” refers to a marker indicative of the state (*e.g.*, presence and/or level) of a RNA (*e.g.*, mRNA) that is present within a biological entity (*e.g.*, a cell or an extracellular vesicle). In many embodiments, an intravesicular RNA biomarker is associated with or present within an extracellular vesicle. In some embodiments, an intravesicular RNA biomarker is associated or specific to cancer. In some embodiments, an intravesicular RNA biomarker is or comprises an mRNA transcript. In some embodiments, an intravesicular RNA biomarker is or comprises a noncoding RNA. Exemplary noncoding RNAs may include, but are not limited to small nuclear RNA, microRNA (miRNA), small nucleolar RNA (snoRNA), circular RNA (circRNA), long noncoding RNA (lncRNA), small noncoding RNA, piwi-interacting RNA, etc.). Certain RNA biomarkers for cancer are described in the art, *e.g.*, as described in Xi *et al.* “RNA Biomarkers: Frontier of Precision Medicine for Cancer” *Noncoding RNA* (2017) 3:9, the contents of which are incorporated herein by reference for purposes described herein. In some embodiments, an intravesicular RNA biomarker is or comprise an orphan noncoding RNA (oncRNA). Certain oncRNAs that are cancer-specific were identified and described in the art, *e.g.*, as described in Teng *et al.* “Orphan noncoding RNAs: novel regulators and cancer biomarkers” *Ann Transl Med* (2019) 7:S21; Fish *et al.* “Cancer cells exploit an orphan RNA to drive metastatic progression” *Nature Medicine* (2018) 24: 1743-1751; International Patent Publication WO 2019/094780, each of which are incorporated herein by reference for purposes described herein. In some embodiments, an intravesicular RNA biomarker is or comprises a long non-coding RNA. Certain non-coding RNA biomarkers for cancer are described in the art, *e.g.*, as described in Qian *et al.* “Long Non-coding RNAs in Cancer: Implications for Diagnosis, Prognosis, and Therapy” *Front. Med.* (2020) Volume 7, Article 612393, the contents of which are incorporated herein by reference for purposes described herein. In some embodiments, an intravesicular RNA biomarker is or comprises piwiRNA. In some embodiments, an intravesicular RNA biomarker is or comprises

miRNA. In some embodiments, an intravesicular RNA biomarker is or comprises snoRNA. In some embodiments, an intravesicular RNA biomarker is or comprises circRNA.

[0289] *Ligase:* As used herein, the term “ligase” or “nucleic acid ligase” refers to an enzyme for use in ligating nucleic acids. In some embodiments, a ligase is enzyme for use in ligating a 3'-end of a polynucleotide to a 5'-end of a polynucleotide. In some embodiments, a ligase is an enzyme for use to perform a sticky-end ligation. In some embodiments, a ligase is an enzyme for use to perform a blunt-end ligation. In some embodiments, a ligase is or comprises a DNA ligase.

[0290] *Life-history-associated risk factors:* As used herein, the term “life-history risk factors” refers to individuals' actions, experiences, medical history, and/or exposures in their lives which may directly or indirectly increase such individuals' risk for a condition, *e.g.*, ovarian cancer, relative to individuals who do not have such actions, experiences, medical history, and/or exposures in their lives. In some embodiments, non-limiting examples of life-history-associated risk factors include smoking, alcohol, drugs, carcinogenic agents, diet, obesity, diabetes, polycystic ovarian syndrome (PCOS), endometriosis, pelvic inflammatory disease (PID), nulliparousness/infertility, no history/short history of oral contraceptive use, physical activity, sun exposure, radiation exposure, perineal talc use, hormone replacement therapy (HRT), exposure to infectious agents such as viruses, and/or occupational hazard (Reid *et al.*, 2017; which is incorporated herein by reference for the purpose described herein). One skilled in the art recognizes that the above list of life-history-associated risk factors contributing to cancer (*e.g.*, ovarian cancer) susceptibility is not exhaustive but constantly evolving.

[0291] *Ligation:* As used herein, the term “ligate”, “ligating” or “ligation” refers to a method or composition known in the art for joining two oligonucleotides or polynucleotides. A ligation may be or comprise a sticky-end ligation or a blunt-end ligation. In some embodiments, ligation involved in provided technologies is or comprises a sticky-end ligation. In some embodiments, ligation refers to joining a 3' end of a polynucleotide to a 5' end of a polynucleotide. In some embodiments, ligation is facilitated by use of a nucleic acid ligase.

[0292] *Nanoparticles:* The term “nanoparticles” as used in the context of a sample for a detection assay (*e.g.*, as described herein) refers to nanoparticles having a size range of interest that includes extracellular vesicles. In some embodiments, such nanoparticles have a size range of about 30 nm to about 1000 nm. In some embodiments, nanoparticles have a size range of about 30 nm to about 750 nm. In some embodiments, nanoparticles have a size range of about 50 nm to about 750 nm. In some embodiments, nanoparticles have a size range of about 30 nm to about 500 nm. In some embodiments, nanoparticles have a size range of about 50 nm to about 500 nm. In some embodiments, nanoparticles described herein are obtained from a bodily fluid sample (*e.g.*, a blood-derived sample) of a subject, for example, in some embodiments by a size exclusion-based method (*e.g.*, in some embodiments size exclusion chromatography). In some embodiments, nanoparticles

are or comprise analyte aggregates, which in some embodiments may be or comprise protein or mucin aggregates. In some embodiments, nanoparticles are or comprise protein multimers. In some embodiments, nanoparticles are or comprise extracellular vesicles. In some embodiments, nanoparticles are or comprise intact extracellular vesicles.

[0293] *Non-cancer subjects:* As used herein, the term “non-cancer subjects” generally refers to female subjects who do not have non-benign ovarian cancer. For example, in some embodiments, a non-cancer subject is a healthy female subject (*e.g.*, a healthy woman subject). In some embodiments, a non-cancer subject is a healthy female subject (*e.g.*, a healthy woman subject) below age 55. In some embodiments, a non-cancer subject is a healthy female subject (*e.g.*, a healthy woman subject) with age 55 or above. In some embodiments, a non-cancer subject is a female subject (*e.g.*, woman subject) with non-ovarian related health diseases, disorders, or conditions. In some embodiments, a non-cancer subject is a female subject (*e.g.*, a woman subject) having a benign ovarian tumor (*e.g.*, a benign mass observed in a fallopian tube and/or on an ovary).

[0294] *Nucleic acid/ Oligonucleotide:* As used herein, the term “nucleic acid” refers to a polymer of at least 10 nucleotides or more. In some embodiments, a nucleic acid is or comprises DNA. In some embodiments, a nucleic acid is or comprises RNA. In some embodiments, a nucleic acid is or comprises peptide nucleic acid (PNA). In some embodiments, a nucleic acid is or comprises a single stranded nucleic acid. In some embodiments, a nucleic acid is or comprises a double-stranded nucleic acid. In some embodiments, a nucleic acid comprises both single and double-stranded portions. In some embodiments, a nucleic acid comprises a backbone that comprises one or more phosphodiester linkages. In some embodiments, a nucleic acid comprises a backbone that comprises both phosphodiester and non-phosphodiester linkages. For example, in some embodiments, a nucleic acid may comprise a backbone that comprises one or more phosphorothioate or 5'-N-phosphoramidite linkages and/or one or more peptide bonds, *e.g.*, as in a “peptide nucleic acid”. In some embodiments, a nucleic acid comprises one or more, or all, natural residues (*e.g.*, adenine, cytosine, deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine, guanine, thymine, uracil). In some embodiments, a nucleic acid comprises one or more, or all, non-natural residues. In some embodiments, a non-natural residue comprises a nucleoside analog (*e.g.*, 2-aminoadenosine, 2-thiiothymidine, inosine, pyrrolo-pyrimidine, 3 -methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5 -propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 6-O-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a non-natural residue comprises one or more modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared to those in natural residues. In some embodiments, a nucleic acid has a nucleotide sequence that encodes a functional gene

product such as an RNA or polypeptide. In some embodiments, a nucleic acid has a nucleotide sequence that comprises one or more introns. In some embodiments, a nucleic acid may be prepared by isolation from a natural source, enzymatic synthesis (*e.g.*, by polymerization based on a complementary template, *e.g.*, *in vivo* or *in vitro*, reproduction in a recombinant cell or system, or chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, or 20,000 or more residues or nucleotides long.

[0295] *Nucleotide:* As used herein, the term “nucleotide” refers to its art-recognized meaning. When a number of nucleotides is used as an indication of size, *e.g.*, of an oligonucleotide, a certain number of nucleotides refers to the number of nucleotides on a single strand, *e.g.*, of an oligonucleotide.

[0296] *Patient:* As used herein, the term “patient” refers to any organism who is suffering or at risk of a disease or disorder or condition. Typical patients include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and/or humans). In some embodiments, a patient is a human. In some embodiments, a patient is suffering from or susceptible to one or more diseases or disorders or conditions. In some embodiments, a patient displays one or more symptoms of a disease or disorder or condition. In some embodiments, a patient has been diagnosed with one or more diseases or disorders or conditions. In some embodiments, a disease or disorder or condition that is amenable to provided technologies is or includes cancer, or presence of one or more tumors. In some embodiments, a patient is receiving or has received certain therapy to diagnose and/or to treat a disease, disorder, or condition.

[0297] *Polypeptide:* The term “polypeptide”, as used herein, typically has its art-recognized meaning of a polymer of at least three amino acids or more. Those of ordinary skill in the art will appreciate that the term “polypeptide” is intended to be sufficiently general as to encompass not only polypeptides having a complete sequence recited herein, but also to encompass polypeptides that represent functional, biologically active, or characteristic fragments, portions or domains (*e.g.*, fragments, portions, or domains retaining at least one activity) of such complete polypeptides. In some embodiments, polypeptides may contain L-amino acids, D-amino acids, or both and/or may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, *etc.* In some embodiments, polypeptides may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof (*e.g.*, may be or comprise peptidomimetics).

[0298] *Prevent or prevention:* As used herein, “prevent” or “prevention,” when used in connection with the occurrence of a disease, disorder, and/or condition, refers to reducing the risk of developing the disease, disorder and/or condition and/or to delaying onset of one or more characteristics or symptoms of the disease, disorder or condition. Prevention may be considered complete when onset of a disease, disorder or condition has been delayed for a predefined period of time.

[0299] *Primer:* As used herein, the term “primer” refers to an oligonucleotide capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced (*e.g.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). A primer is preferably single stranded for maximum efficiency in amplification. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of a primer can depend on many factors, *e.g.*, desired annealing temperature, etc.

[0300] *Reference:* As used herein, “reference” describes a standard or control relative to which a comparison is performed. For example, in some embodiments, an agent, animal, individual, population, sample, sequence or value of interest is compared with a reference or control agent, animal, individual, population, sample, sequence, or value. In some embodiments, a reference or control is tested and/or determined substantially simultaneously with the testing or determination of interest. In some embodiments, a reference or control is a historical reference or control, optionally embodied in a tangible medium. In some embodiments, a reference or control in the context of a reference level of a target refers to a level of a target in a normal healthy subject or a population of normal healthy subjects. In some embodiments, a reference or control in the context of a reference level of a target refers to a level of a target in a subject prior to a treatment. Typically, as would be understood by those skilled in the art, a reference or control is determined or characterized under comparable conditions or circumstances to those under assessment. Those skilled in the art will appreciate when sufficient similarities are present to justify reliance on and/or comparison to a particular possible reference or control.

[0301] *Risk:* As will be understood from context, “risk” of a disease, disorder, and/or condition refers to a likelihood that a particular individual will develop the disease, disorder, and/or condition. In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 up to 100%. In some embodiments risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference samples have a known risk of a disease, disorder, condition and/or event. In some embodiments a reference sample or group of

reference samples are from individuals comparable to a particular individual. In some embodiments, relative risk is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

[0302] *Sample:* As used herein, the term “sample” typically refers to an aliquot of material obtained or derived from a source of interest. In some embodiments, a sample is obtained or derived from a biological source (*e.g.*, a tissue or organism or cell culture) of interest. In some embodiments, a source of interest may be or comprise a cell or an organism, such as an animal or human. In some embodiments, a source of interest is or comprises biological tissue or fluid. In some embodiments, a biological tissue or fluid may be or comprise amniotic fluid, aqueous humor, ascites, bile, bone marrow, blood, breast milk, cerebrospinal fluid, cerumen, chyle, chime, ejaculate, endolymph, exudate, feces, gastric acid, gastric juice, lymph, mucus, pericardial fluid, perilymph, peritoneal fluid, pleural fluid, pus, rheum, saliva, sebum, semen, serum, smegma, sputum, synovial fluid, sweat, tears, urine, vaginal secretions, vitreous humor, vomit, and/or combinations or component(s) thereof. In some embodiments, a biological fluid may be or comprise an intracellular fluid, an extracellular fluid, an intravesicular fluid (blood plasma), an interstitial fluid, a lymphatic fluid, and/or a transcellular fluid. In some embodiments, a biological tissue or sample may be obtained, for example, by aspirate, biopsy (*e.g.*, fine needle or tissue biopsy), swab (*e.g.*, oral, nasal, skin, or vaginal swab), scraping, surgery, washing or lavage (*e.g.*, bronchoalveolar, ductal, nasal, ocular, oral, uterine, vaginal, or other washing or lavage). In some embodiments, a biological sample is or comprises a bodily fluid sample or a bodily fluid-derived sample. Examples of a bodily fluid include, but are not limited to an amniotic fluid, bile, blood, breast milk, bronchoalveolar lavage fluid (BAL), cerebrospinal fluid, dialysate, feces, saliva, semen, synovial fluid, tears, urine, etc. In some embodiments, a biological sample is or comprises a liquid biopsy. In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, a sample is a “primary sample” obtained directly from a source of interest by any appropriate means. In some embodiments, as will be clear from context, the term “sample” refers to a preparation that is obtained by processing (*e.g.*, by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, a sample is a preparation that is processed by using a semi-permeable membrane or an affinity-based method such antibody-based method to separate a biological entity of interest from other non-target entities. Such a “processed sample” may comprise, for example, in some embodiments, nanoparticles, while, in some embodiments, nucleic acids and/or proteins, *etc.*, extracted from a sample. In some embodiments, a processed sample can be obtained by subjecting a primary sample to one or more techniques such as amplification or reverse transcription of nucleic acid, isolation and/or purification of certain components, *etc.*

[0303] *Selective or specific:* The term “selective” or “specific”, when used herein with reference to an agent having an activity, is understood by those skilled in the art to mean that the agent discriminates between potential target entities, states, or cells. For example, in some

embodiments, an agent is said to bind “specifically” to its target if it binds preferentially with that target in the presence of one or more competing alternative targets. In many embodiments, specific interaction is dependent upon the presence of a particular structural feature of the target entity (*e.g.*, an epitope, a cleft, a binding site). It is to be understood that specificity need not be absolute. In some embodiments, specificity may be evaluated relative to that of a target-binding moiety for one or more other potential target entities (*e.g.*, competitors). In some embodiments, specificity is evaluated relative to that of a reference specific binding moiety. In some embodiments, specificity is evaluated relative to that of a reference non-specific binding moiety. In some embodiments, a target-binding moiety does not detectably bind to the competing alternative target under conditions of binding to its target entity. In some embodiments, a target-binding moiety binds with higher on-rate, lower off-rate, increased affinity, decreased dissociation, and/or increased stability to its target entity as compared with the competing alternative target(s).

[0304] *Small molecule:* As used herein, the term “small molecule” means a low molecular weight organic and/or inorganic compound. In general, a “small molecule” is a molecule that is less than about 5 kilodaltons (kD) in size. In some embodiments, a small molecule is less than about 4 kD, 3 kD, about 2 kD, or about 1 kD. In some embodiments, the small molecule is less than about 800 Daltons (D), about 600 D, about 500 D, about 400 D, about 300 D, about 200 D, or about 100 D. In some embodiments, a small molecule is less than about 2000 g/mol, less than about 1500 g/mol, less than about 1000 g/mol, less than about 800 g/mol, or less than about 500 g/mol. In some embodiments, a small molecule is not a polymer. In some embodiments, a small molecule does not include a polymeric moiety. In some embodiments, a small molecule is not a protein or polypeptide (*e.g.*, is not an oligopeptide or peptide). In some embodiments, a small molecule is not a polynucleotide (*e.g.*, is not an oligonucleotide). In some embodiments, a small molecule is not a polysaccharide. In some embodiments, a small molecule does not comprise a polysaccharide (*e.g.*, is not a glycoprotein, proteoglycan, glycolipid, *etc.*). In some embodiments, a small molecule is not a lipid. In some embodiments, a small molecule is biologically active. In some embodiments, suitable small molecules may be identified by methods such as screening large libraries of compounds (Beck-Sickinger & Weber (2001) *Combinational Strategies in Biology and Chemistry* (John Wiley & Sons, Chichester, Sussex); by structure-activity relationship by nuclear magnetic resonance (Shuker *et al.* (1996) "Discovering high-affinity ligands for proteins: SAR by NMR." *Science* 274: 1531-1534); encoded self-assembling chemical libraries (Melkko *et al.* (2004) "Encoded self-assembling chemical libraries." *Nature Biotechnol.* 22: 568-574); DNA-templated chemistry (Gartner *et al.* (2004) "DNA-templated organic synthesis and selection of a library of macrocycles." *Science* 305: 1601-1605); dynamic combinatorial chemistry (Ramstrom & Lehn (2002) "Drug discovery by dynamic combinatorial libraries." *Nature Rev. Drug Discov.* 1: 26-36); tethering (Arkin & Wells (2004) "Small-molecule inhibitors of protein-protein interactions: progressing towards the dream."

Nature Rev. Drug Discov. 3: 301-317); and speed screen (Muckenschnabel *et al.* (2004)

"SpeedScreen: label-free liquid chromatography-mass spectrometry-based high- throughput screening for the discovery of orphan protein ligands." *Anal. Biochem.* 324: 241-249). In some embodiments, a small molecule may have a dissociation constant for a target in the nanomolar range.

[0305] *Specific binding:* As used herein, the term "specific binding" refers to an ability to discriminate between possible binding partners in the environment in which binding is to occur. A target-binding moiety that interacts with one particular target when other potential targets are present is said to "bind specifically" to the target with which it interacts. In some embodiments, specific binding is assessed by detecting or determining degree of association between a target-binding moiety and its partner; in some embodiments, specific binding is assessed by detecting or determining degree of dissociation of a target-binding moiety-partner complex; in some embodiments, specific binding is assessed by detecting or determining ability of a target-binding moiety to compete an alternative interaction between its partner and another entity. In some embodiments, specific binding is assessed by performing such detections or determinations across a range of concentrations.

[0306] *Stage of cancer:* As used herein, the term "stage of cancer" refers to a qualitative or quantitative assessment of the level of advancement of a cancer (*e.g.*, ovarian cancer). In some embodiments, criteria used to determine the stage of a cancer may include, but are not limited to, one or more of where the cancer is located in a body, tumor size, whether the cancer has spread to lymph nodes, whether the cancer has spread to one or more different parts of the body, *etc.* In some embodiments, cancer may be staged using the AJCC staging system. The AJCC staging system is a classification system, developed by the American Joint Committee on Cancer for describing the extent of disease progress in cancer patients, which utilizes in part the TNM scoring system: Tumor size, Lymph Nodes affected, Metastases. In some embodiments, cancer may be staged using a classification system that in part involves the TNM scoring system, according to which T refers to the size and extent of the main tumor, usually called the primary tumor; N refers to the number of nearby lymph nodes that have cancer; and M refers to whether the cancer has metastasized. In some embodiments, a cancer may be referred to as Stage 0 (abnormal cells are present but have not spread to nearby tissue, also called carcinoma *in situ*, or CIS; CIS is not cancer, but it may become cancer), Stage I-III (cancer is present; the higher the number, the larger the tumor and the more it has spread into nearby tissues), or Stage IV (the cancer has spread to distant parts of the body). In some embodiments, a cancer may be assigned to a stage selected from the group consisting of: *in situ* (abnormal cells are present but have not spread to nearby tissue); localized (cancer is limited to the place where it started, with no sign that it has spread); regional (cancer has spread to nearby lymph nodes, tissues, or organs); distant (cancer has spread to distant parts of the body); and unknown (there is not enough information to figure out the stage).

[0307] ***Subject:*** As used herein, the term “subject” refers to an organism from which a sample is obtained, *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, domestic pets, *etc.*) and humans. In some embodiments, a subject is a human female subject, *e.g.*, a human woman subject. In some embodiments, a subject is suffering from ovarian cancer. In some embodiments, a subject is susceptible to ovarian cancer. In some embodiments, a subject displays one or more symptoms or characteristics of ovarian cancer. In some embodiments, a subject displays one or more non-specific symptoms of ovarian cancer. In some embodiments, a subject does not display any symptom or characteristic of ovarian cancer. In some embodiments, a subject is someone with one or more features characteristic of susceptibility to or risk of ovarian cancer. In some embodiments, a subject is a patient. In some embodiments, a subject is an individual to whom diagnosis and/or therapy is and/or has been administered. In some embodiments, a subject is a female subject (*e.g.*, woman subject) determined to have an adnexal mass. In some embodiments, a subject is an asymptomatic subject. Such an asymptomatic subject may be a female subject (*e.g.*, woman subject) at average population risk or with hereditary risk. For example, 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 embodiments, 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. In some embodiments, a subject amenable to provided technologies is an individual selected based on one or more characteristics such as age, race, geographic location, 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).

[0308] ***Suffering from:*** An individual who is “suffering from” a disease, disorder, and/or condition has been diagnosed with and/or displays one or more symptoms of a disease, disorder, and/or condition.

[0309] ***Surface analyte:*** As used herein, a “surface analyte” refers to an analyte present on the surface of a biological entity (*e.g.*, a cell or a nanoparticle from a biological sample). In some embodiments, a surface analyte is or comprises a surface polypeptide or surface protein. In some embodiments, a surface analyte is or comprises a glycan.

[0310] ***Surface biomarker:*** As used herein, a “surface biomarker” refers to a marker indicative of the state (*e.g.*, presence, level, and/or activity) of a surface analyte (*e.g.*, as described herein) of a biological entity (*e.g.*, a cell or a nanoparticle including, *e.g.*, in some embodiments an analyte aggregate (*e.g.*, a protein or mucin aggregate) and/or an extracellular vesicle). In some

embodiments, a surface biomarker is or comprises a surface protein biomarker. In some embodiments, a surface biomarker is or comprises a carbohydrate-dependent marker.

[0311] *Surface polypeptide or surface protein:* As used interchangeably herein, the terms “surface polypeptide,” and “surface protein” refer to a polypeptide or protein present in and/or on the surface of a biological entity (e.g., a cell or a nanoparticle including, e.g., in some embodiments an analyte aggregate (e.g., a protein or mucin aggregate) and/or an extracellular vesicle, etc.) through direct or indirect interactions. As will be understood by a skilled artisan, a surface protein, in some embodiments, may comprise a post-translational modification, including, e.g., but not limited to glycosylation. In some embodiments, a surface polypeptide or protein may be or comprise a membrane-bound polypeptide. In some embodiments, a membrane-bound polypeptide refers to a polypeptide or protein with one or more domains or regions present in and/or on the surface of the membrane of a biological entity (e.g., a cell, an extracellular vesicle, etc.). In some embodiments, a membrane-bound polypeptide may comprise one or more domains or regions spanning and/or associated with the plasma membrane of a biological entity (e.g., a cell, an extracellular vesicle, etc.). In some embodiments, a -bound polypeptide may comprise one or more domains or regions spanning and/or associated with the plasma membrane of a biological entity (e.g., a cell, an extracellular vesicle, etc.) and also protruding into the intracellular and/or intravesicular space. In some embodiments, a membrane-bound polypeptide may comprise one or more domains or regions associated with the plasma membrane of a biological entity (e.g., a cell, an extracellular vesicle, etc.), for example, via one or more non-peptidic linkages (e.g., through a glycosylphosphatidylinositol (GPI) anchor or lipidification or through non-covalent interaction). In some embodiments, a membrane-bound polypeptide may comprise one or more domains or regions that is/are anchored into either side of plasma membrane of a biological entity (e.g., a cell, an extracellular vesicle, etc.). In some embodiments, a surface protein is associated with or present on the surface of a nanoparticle (e.g., as described herein). In some embodiments, a surface protein is associated with or present within an extracellular vesicle. In some embodiments, a surface protein may be associated with or present within a ovarian cancer-associated extracellular vesicle (e.g., an extracellular vesicle obtained or derived from a bodily fluid-derived sample (e.g., but not limited to a blood-derived sample) of a subject suffering from or susceptible to ovarian cancer). As will be understood by a skilled artisan, detection of the presence of at least a portion of a surface polypeptide or surface protein on/within extracellular vesicles can facilitate separation and/or isolation of ovarian cancer-associated extracellular vesicles from a biological sample

(e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) (e.g., a blood or blood-derived sample) from a subject. In some embodiments, detection of the presence of a surface polypeptide or surface protein may be or comprise detection of an intravesicular portion (e.g., an intravesicular epitope) of such a surface polypeptide or surface protein. In some embodiments, detection of the presence of a surface polypeptide or surface protein may be or comprise detection of a membrane-spanning portion of such a surface polypeptide or surface protein. In some embodiments, detection of the presence of a surface polypeptide or surface protein may be or comprise detection of an extravesicular portion of such a surface polypeptide or surface protein...

[0312] ***Surface protein biomarker:*** As used herein, the term “surface protein biomarker” refers to a marker indicative of the state (e.g., presence, level, and/or activity) of a surface protein (e.g., as described herein) of a biological entity (e.g., a cell or a nanoparticle including, e.g., in some embodiments an analyte aggregate (e.g., a protein or mucin aggregate) and/or an extracellular vesicle). In some embodiments, a surface protein refers to a polypeptide or protein with one or more domains or regions located in or on the surface of the membrane of a biological entity (e.g., a cell or an extracellular vesicle). In some embodiments, a surface protein biomarker may be or comprise an epitope that is present on the interior side (intravesicular) or the exterior side (extravesicular) of the membrane. In some embodiments, a surface protein biomarker is associated with or present in an extracellular vesicle. In some embodiments, a surface protein biomarker may be or comprise a mutated polypeptide. In some embodiments, a surface protein biomarker may be post-translationally modified (e.g., but not limited to glycosylated, phosphorylated, etc.). In some embodiments, a surface protein biomarker may be post-translationally processed and present in the form of a truncated polypeptide, for example, as a result of proteolytic cleavage). In some embodiments, a surface-protein biomarker may be or comprise an epitope that is present on the exterior surface of a nanoparticle.

[0313] ***Susceptible to:*** An individual who is “susceptible to” a disease, disorder, and/or condition is one who has a higher risk of developing the disease, disorder, and/or condition than does a member of the general public. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may not have been diagnosed with the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may not exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0314] *Target-binding moiety:* In general, the terms “target-binding moiety” and “binding moiety” are used interchangeably herein to refer to any entity or moiety that binds to a target of interest (*e.g.*, molecular target of interest such as a biomarker or an epitope). In many embodiments, a target-binding moiety of interest is one that binds specifically with its target (*e.g.*, a target biomarker) in that it discriminates its target from other potential binding partners in a particular interaction context. In general, a target-binding moiety may be or comprise an entity or moiety of any chemical class (*e.g.*, polymer, non-polymer, small molecule, polypeptide, carbohydrate, lipid, nucleic acid, *etc.*). In some embodiments, a target-binding moiety is a single chemical entity. In some embodiments, a target-binding moiety is a complex of two or more discrete chemical entities associated with one another under relevant conditions by non-covalent interactions. For example, those skilled in the art will appreciate that in some embodiments, a target-binding moiety may comprise a “generic” binding moiety (*e.g.*, one of biotin/avidin/streptavidin and/or a class-specific antibody) and a “specific” binding moiety (*e.g.*, an antibody or aptamers with a particular molecular target) that is linked to the partner of the generic binding moiety. In some embodiments, such an approach can permit modular assembly of multiple target binding moieties through linkage of different specific binding moieties with a generic binding moiety partner.

[0315] *Target biomarker signature:* The term “target biomarker signature”, as used herein, refers to a combination of (*e.g.*, at least 2 or more, including, *e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, or more) biomarkers, which combination correlates with a particular biological event or state of interest, so that one skilled in the art will appreciate that it may appropriately be considered to be a “signature” of that event or state. To give but a few examples, in some embodiments, a target biomarker signature may correlate with a particular disease or disease state, and/or with likelihood that a particular disease, disorder or condition may develop, occur, or reoccur. In some embodiments, a target biomarker signature may correlate with a particular disease or therapeutic outcome, or likelihood thereof. In some embodiments, a target biomarker signature may correlate with a specific cancer and/or stage thereof. In some embodiments, a target biomarker signature may correlate with ovarian cancer and/or a stage and/or a subtype thereof. In some embodiments, a target biomarker signature comprises a combination of (*e.g.*, at least 2 or more, including, *e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, or more) biomarkers that together are specific for an ovarian cancer or a subtype and/or a disease stage thereof), though one or more biomarkers in such a combination may be directed to a target (*e.g.*, a surface protein biomarker, an intravesicular protein biomarker, and/or an intravesicular RNA) that is not specific to the ovarian cancer. For example, in some embodiments, a target biomarker signature

may comprise at least one biomarker specific to an ovarian cancer or a stage and/or subtype thereof (*i.e.*, an ovarian cancer-specific target), and may further comprise a biomarker that is not necessarily or completely specific for the ovarian cancer (*e.g.*, that may also be found on some or all biological entities such as, *e.g.*, cells, nanoparticles, *etc.*, that are not cancerous, are not of the relevant cancer, and/or are not of the particular stage and/or subtype of interest). That is, as will be appreciated by those skilled in the art reading the present specification, so long as a combination of biomarkers utilized in a target biomarker signature is or comprises a plurality of biomarkers that together are specific for the relevant target biological entities of interest (*e.g.*, ovarian cancer cells of interest or nanoparticles secreted by ovarian cancer cells) (*i.e.*, sufficiently distinguish the relevant target biological entities (*e.g.*, ovarian cancer cells of interest or nanoparticles secreted by ovarian cancer cells) for detection from other biological entities not of interest for detection), such a combination of biomarkers is a useful target biomarker signature in accordance with certain embodiments of the present disclosure.

[0316] *Therapeutic agent:* As used interchangeably herein, the phrase “therapeutic agent” or “therapy” refers to an agent or intervention that, when administered to a subject or a patient, has a therapeutic effect and/or elicits a desired biological and/or pharmacological effect. In some embodiments, a therapeutic agent or therapy is any substance that can be used to alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. In some embodiments, a therapeutic agent or therapy is a medical intervention (*e.g.*, surgery, radiation, phototherapy) that can be performed to alleviate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition.

[0317] *Threshold level (e.g., cutoff):* As used herein, the term “threshold level” refers to a level that are used as a reference to attain information on and/or classify the results of a measurement, for example, the results of a measurement attained in an assay. For example, in some embodiments, a threshold level (*e.g.*, a cutoff) means a value measured in an assay that defines the dividing line between two subsets of a population (*e.g.*, normal and/or non-ovarian cancer vs. ovarian cancer). Thus, a value that is equal to or higher than the threshold level defines one subset of the population, and a value that is lower than the threshold level defines the other subset of the population. A threshold level can be determined based on one or more control samples or across a population of control samples. A threshold level can be determined prior to, concurrently with, or after the measurement of interest is taken. In some embodiments, a threshold level can be a range of values.

[0318] *Treat:* As used herein, the term “treat,” “treatment,” or “treating” refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease,

disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition. In some embodiments, treatment may be administered to a subject who exhibits only early signs of the disease, disorder, and/or condition, for example for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition. In some embodiments, treatment may be administered to a subject at a later-stage of disease, disorder, and/or condition.

[0319] Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (*e.g.*, electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference for the purpose described herein.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0320] Ovarian cancer was responsible for an estimated 14,070 deaths in 2018 in the United States (Torre *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). The majority of these deaths are attributable to late diagnosis; ovarian cancer has an estimated five-year survival rate of 93% if caught at its earliest stage versus 26% if caught at its latest stage (Torre *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). The detection of high-grade serous ovarian cancer (HGSOC) is particularly important given that HGSOC accounts for 70% to 80% of all ovarian cancer deaths, while other subtypes are slower growing and susceptible to over diagnosis when using current technologies (Temkin *et al.*, 2017; which is incorporated herein by reference for the purpose described herein). Unfortunately, despite being the fifth largest killer of women among all cancers (Howlader *et al.*, 2019; which is incorporated herein by reference for the purpose described herein), there are no recommended ovarian cancer screening tests for average-risk women. While many women at hereditary risk and/or who may be experiencing one or more symptoms of ovarian cancer (*e.g.*, fluid in the peritoneal cavity (ascites), general gastrointestinal dysfunction, constipation, bowel obstruction, nausea, vomiting, diarrhea, gastrointestinal reflux, increased abdominal size, urinary symptoms, abdominal bloating, abdominal and/or pelvic pain, fatigue, and/or shortness of breath) are currently screened by plasma CA-125 and/or transvaginal ultrasound (TVUS), these tests are suboptimal for screening, because they have low sensitivity (~20%) for stage I and II disease and poor specificity. For example, the Prostate, Lung, Colorectal and Ovarian Cancer Screening Randomized Trial found

plasma CA-125 and TVUS increases the number of unnecessary surgeries and provides no mortality benefit for average-risk women (Buys *et al.*, 2011; which is incorporated herein by reference for the purpose described herein). Despite this poor performance, plasma CA-125 and TVUS are currently common screening tools for triaging post-menopausal women with nonspecific pelvic pain, which may be potentially indicative of ovarian cancer.

[0321] The present disclosure, among other things, identifies the source of a problem with certain prior technologies including, for example, certain conventional approaches to detection and diagnosis of ovarian cancer. For example, the present disclosure appreciates that many conventional diagnostic assays, *e.g.*, based on cell-free nucleic acids, serum proteins (*e.g.*, CA-125), 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 embodiments, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by identification of biomarker combinations that are predicted to exhibit high sensitivity and specificity for ovarian cancer based on bioinformatics analysis. In some embodiments, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, by detecting co-localization of a target biomarker signature of ovarian cancer (*e.g.*, identified by bioinformatics analysis) in individual nanoparticles, which comprises at least one extracellular vesicle-associated surface biomarker and at least one target biomarker comprising a target surface marker, which may be a polypeptide or a carbohydrate-dependent marker, present in nanoparticles associated with ovarian cancer. In some embodiments, a target biomarker signature may further comprise at least one internal biomarker (*e.g.*, internal protein biomarkers as described herein, and/or RNA biomarkers as described herein) present in nanoparticles associated with ovarian cancer. In some embodiments, the present disclosure provides technologies (including systems, compositions, and methods) that solve such problems, among other things, by detecting such target biomarker signature of ovarian cancer using a target entity detection approach that was developed by Applicant and described in US 2020/0299780 and WO 2020/180741, which are based on interaction and/or co-localization of a target biomarker signature in individual nanoparticles. The contents of each of the aforementioned disclosures are incorporated herein by reference in their entirety.

[0322] In some embodiments, extracellular vesicles for detection as described herein can be isolated from a bodily fluid of a subject by a size exclusion-based method. As will be understood by a skilled artisan, in some embodiments, a size exclusion-based method may provide a sample comprising nanoparticles having a size range of interest that includes extracellular vesicles. Accordingly, in some embodiments, provided technologies of the present disclosure encompass detection, in individual nanoparticles having a size range of interest (*e.g.*, in some embodiments about 30 nm to about 1000 nm) that includes extracellular vesicles, of co-localization of at least two

or more surface biomarkers (e.g., as described herein) that forms a target biomarker signature of ovarian cancer. A skilled artisan reading the present disclosure will understand that various embodiments described herein in the context of “extracellular vesicle(s)” (e.g., assays for detecting individual extracellular vesicles and/or provided “extracellular vesicle-associated surface biomarkers”) can be also applicable in the context of “nanoparticles” as described herein.

[0323] The present disclosure, among other things, provides insights and technologies for achieving effective ovarian cancer screening, *e.g.*, for early detection of ovarian cancer. In some embodiments, the present disclosure provides technologies for early detection of ovarian cancer in women who may be experiencing one or more symptoms associated with ovarian cancer. In some embodiments, the present disclosure provides technologies for early detection of ovarian cancer in women who are at hereditary risks for ovarian cancer. In some embodiments, the present disclosure provides technologies for early detection of ovarian cancer in post-menopausal women who may be at hereditary risk and/or experiencing one or more symptoms associated with ovarian cancer. In some embodiments, the present disclosure provides technologies for screening women at hereditary or average risk for early-stage high-grade serous ovarian cancer (HGSOC). HGSOC is the most common and lethal subtype of ovarian cancer, in which 84% of cases are detected at an advanced stage (Torre *et al.*, 2018, which is incorporated herein by reference for the purpose described herein). In some embodiments, provided technologies are effective for detection of early-stage ovarian cancers. In some embodiments, provided technologies are effective even when applied to populations comprising or consisting of asymptomatic or symptomatic individuals (*e.g.*, due to sufficiently high sensitivity and/or low rates of false positive and/or false negative results). In some embodiments, provided technologies are effective when applied to populations comprising or consisting of individuals (*e.g.*, asymptomatic, or symptomatic individuals) without hereditary risk in developing ovarian cancer. In some embodiments, provided technologies are effective when applied to populations comprising or consisting of individuals (*e.g.*, asymptomatic, or symptomatic individuals) with hereditary risk in developing ovarian cancer. In some embodiments, provided technologies are effective when applied to populations comprising or consisting of individuals susceptible to ovarian cancer (*e.g.*, individuals with a known genetic, environmental, or experiential risk, *etc.*). In some embodiments, provided technologies may be or include one or more compositions (*e.g.*, molecular complexes, systems, 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.

[0324] In some embodiments, 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 ovarian cancer, 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 embodiments, provided technologies are useful in conjunction with an individual's regular medical examinations, such as but not limited to physicals, general practitioner visits, cholesterol/lipid blood tests, diabetes (type 2) screening, colonoscopies, blood pressure screening, thyroid function tests, prostate cancer screening, mammograms, HPV/Pap smears, and/or vaccinations. In some embodiments, provided technologies are useful in conjunction with other diagnostics assays for ovarian cancer, including, *e.g.*, imaging tests such as abdominal/transvaginal ultrasound, and/or serum biomarkers (*e.g.*, CA-125)).

[0325] In some embodiments, the present disclosure, among other things, provides insights that screening of asymptomatic 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 ovarian cancer. In some embodiments, the present disclosure provides ovarian cancer screening systems that can be implemented to detect ovarian cancer, including early-stage cancer, in some embodiments in asymptomatic individuals (*e.g.*, without hereditary risks in ovarian cancer). In some embodiments, provided technologies are implemented to achieve regular screening of asymptomatic individuals (*e.g.*, with or without hereditary risk(s) in ovarian cancer). In some embodiments, provided technologies are implemented to achieve regular screening of symptomatic individuals (*e.g.*, with or without hereditary risk(s) in ovarian cancer). 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.

I. Ovarian Cancer Detection

[0326] Today there is no ovarian cancer screening test of any kind that is FDA approved for asymptomatic women of average risk, while in the US the average lifetime risk of developing ovarian cancer is 1.3%, the equivalent of 1 in 78 women. The overall ovarian cancer prevalence in the US was 5.7 per 10,000 women aged 55 to 74 years (Buys *et al.*, 2011; which is incorporated herein by reference for the purpose described herein). In 2018, there were approximately 22,240 new cases of ovarian cancer diagnosed and 14,070 ovarian cancer deaths in the US (Torre *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). Among others, age and Menopausal state have been identified as a risk factor for ovarian cancer, where the mean age of initial presentation is approximately 68 years.

[0327] Epithelial ovarian cancer subtypes account for 90% of all ovarian cancers. Epithelial cancers are classified as serous (52%), endometrioid (10%), mucinous (6%), or clear-cell (6%), (Torre *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). Most

serous carcinomas are diagnosed at stage III (51%) or stage IV (29%), when the 5-year survival rate is 42% and 26%, respectively, indicating the need for an early-stage screening test. Germ cell and sex cord-stromal tumors make up the majority of non-epithelial cancers, but account for only 3% and 2%, respectively, of all ovarian cancers. Ovarian cancer affects women of all ethnicities.

[0328] The strongest risk factor for ovarian cancer is a family history of breast or ovarian cancer. Risk of developing invasive epithelial ovarian cancer is increased by approximately 50% among women with a first-degree relative with a history of ovarian cancer, and by 10% with a first-degree relative with breast cancer. Approximately 18% of epithelial ovarian cancer cases, particularly high-grade serous carcinomas, are estimated to be due to inherited mutations that confer elevated risk. Mutations in BRCA1 and BRCA2 account for almost 40% of ovarian cancer cases in women with a family history of the disease. Among women with BRCA1 or BRCA2 mutations, the risk of developing ovarian cancer by age 80 is 44% and 17%, respectively. Rare moderate-penetrance gene mutations for epithelial ovarian cancer include genes that are involved in the Fanconi anemia/BRCA pathway such as PALB2, BARD1, BRIP1, RAD51C, and RAD51D, for example, as described in Matulonis *et al.*, 2016, which is incorporated herein by reference for the purpose described herein. Families with Lynch syndrome are characterized by a germline mutation in a DNA mismatch repair gene (*e.g.*, MLH1, MSH2, MSH6 or PMS2). Women with Lynch syndrome have approximately an 8% risk of developing ovarian cancer (usually non-serous epithelial tumors) by age 70 compared to 0.7% in the general population (Torre, *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). Inherited mutations in other genes involved in DNA repair, such as CHEK2, MRE11A, RAD50, ATM, and TP53 may also increase the risk of developing ovarian cancer. Additional common, low penetrance alleles may also be associated with epithelial ovarian cancer susceptibility as suggested by genome wide association studies. Such genes and loci include: WNT4, RSP01, BCL2L11, HOXD3, HAGLR, TIPARP, SYNPO2, TERT, GPX6, CHMP4C, LINC00824, COL15A1, SMC2-AS1, MLLT10, INCENP, RCCD1, ATAD5, HNF1B, PLEKHM1, SKAP1, ANKLE1, GATAD2A, Cytobands and SNPs 2q13 rs752590, 4q32.3 rs4691139, 9p22 rs3814113, 9q34.2 rs635634, 10p11.21 rs1192691, and/or 19q13.2 rs688187 (Reid *et al.*, 2017; which is incorporated herein by reference for the purpose described herein).

[0329] The number of younger women identified with hereditary risk is expected to increase in the coming years. The NCCN guidelines for pancreatic cancer were updated in December 2019 to include a recommendation to test all patients for germline mutations in ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11 and TP53. Given the overlap of this gene list with the genes conferring hereditary risk for ovarian cancer, it is likely that more daughters of pancreatic patients will become aware of their own genetic risk for both pancreatic and ovarian cancer, moving them from the general risk category into the hereditary risk category. In

addition, a recent cost effectiveness study in breast cancer patients concluded that it is cost effective to screen all breast cancer patients in the US and the UK for germline mutations in BRCA1 and/or BRCA2 and PALB2 (Sun, *et al.*, 2019; which is incorporated herein by reference for the purpose described herein). Implementation of germline genetic testing for all women with breast cancer into practice guidelines will identify additional risk-mutation carriers whose daughters are also at hereditary risk for breast and ovarian cancer. Currently, there is no recommended screening test for ovarian cancer in women (*e.g.*, without hereditary risk). Among other things, in certain embodiments the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay (*e.g.*, as described herein) that can be utilized to provide an ovarian cancer risk assessment. In certain embodiments, assays and/or technologies described herein can provide a score relative to a reference threshold (*e.g.*, as described herein). In certain embodiments, such a score can be or comprise an ovarian cancer risk score. In some embodiments, such a score can be used in conjunction with other ovarian cancer screening assessment(s) such as, *e.g.*, but not limited to CA-125 measurements (*e.g.*, CA-125 serum level measurements and/or TVUS) and/or ovarian cancer-associated risk factor(s) to provide an overall assessment.

[0330] The Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO), which assessed the use of transvaginal ultrasound (TVUS) and a fixed cut-point (≥ 35 U/mL) in the tumor marker CA-125 for early detection, did not observe a reduction in ovarian cancer mortality after up to 19 years of follow-up. The UK Collaborative Trial of Ovarian Cancer Screening evaluated TVUS combined with a risk algorithm incorporating changes in CA-125 levels and found reduced mortality in average-risk women after 15 years. Despite the contradiction, the U.S. Preventive Services Task Force (USPSTF) continues to recommend against screening for ovarian cancer in the general population, concluding that there is adequate evidence that annual screening does not reduce ovarian cancer mortality and can lead to important harms, mainly surgical interventions in women without ovarian cancer.

[0331] Among other things, in certain embodiments the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening women with a hereditary risk for ovarian cancer and/or women who may be experiencing one or more symptoms associated with ovarian cancer. In certain embodiments, the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening symptomatic or asymptomatic women *e.g.*, prior to other screening methods, *e.g.*, TVUS. In certain embodiments, the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening asymptomatic women *e.g.*, prior to other screening methods, *e.g.*, TVUS. In certain embodiments, the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening women with an average risk for ovarian cancer. In certain embodiments, the present disclosure

provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening women with life-history associated risk of ovarian cancer. In certain embodiments, the present disclosure provides an insight that there is a need for development of an ovarian cancer liquid biopsy assay for screening women who are post-menopausal, *e.g.*, post-menopausal women who may be experiencing one or more symptoms associated with ovarian cancer. Despite being the fifth largest killer of women among all cancers (Howlader *et al.*, 2019; which is incorporated herein by reference for the purpose described herein), there is currently no recommended ovarian cancer screening tool for average-risk women, while the current standard of care screening assays (*e.g.*, TVUS and serum marker CA-125 levels) for stage I and II disease in women at hereditary risk and/or women who may be experiencing symptoms of ovarian cancer exhibit low sensitivity (~20%) and low specificity (NCCN, 2019; Buys *et al.*, 2011; which are each incorporated herein by reference for the purpose described herein). These low rates of sensitivity and specificity pose a barrier to efficient and timely diagnosis. Given the incidence of ovarian cancer in average-risk women, inadequate test specificities (*e.g.*, <99.5%) result in false positive results that outnumber true positives by more than an order of magnitude. This places a significant burden on the healthcare system and on the women being screened as false positive results lead to additional tests, unnecessary surgeries, and emotional/physical distress (Buys *et al.*, 2011; which is incorporated herein by reference for the purpose described herein).

[0332] In some embodiments, the present disclosure provides an insight that a particularly useful ovarian cancer screening test may be characterized by: (1) ultrahigh specificity (>98%) to minimize the number of false positives, and (2) high sensitivity (>40%) for stage I and II ovarian cancer (*i.e.*, when prognosis is most favorable). For example, in some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of >98% and a sensitivity of >50%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of >98% and a sensitivity of >60%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of >98% and a sensitivity of >70%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of >99.5% and a sensitivity of >65%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of >99.5% and a sensitivity of >60%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of at least 99% and a sensitivity of at least 70%, for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test (*e.g.*, comprising one or more biomarker combinations described herein, *e.g.*, as shown in **Table 8**) may be characterized by a specificity of at least 90% (including,

e.g., at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or higher) and a sensitivity of at least 80% (including, *e.g.*, at least 85%, at least 90%, at least 95%, or higher), for example, for stage I and II ovarian cancer. In some embodiments, a particularly useful ovarian cancer screening test may be characterized by a specificity of about 90% to about 100% and a sensitivity of about 80% to about 100%, or about 80% to about 95%.

[0333] In some embodiments, the present disclosure provides an insight that a particularly useful ovarian cancer screening test (*e.g.*, in some embodiments comprising one or more biomarker combinations described herein, *e.g.*, as shown in **Table 8**) to differentiate a benign adnexal mass from ovarian cancer that may be characterized by: (1) high specificity (>90%) to minimize the number of false positives, and (2) high sensitivity (>65%) to minimize the number of false negatives. For example, in some embodiments, a particularly useful ovarian cancer screening test (*e.g.*, in some embodiments comprising one or more biomarker combinations described herein, *e.g.*, as shown in **Table 8**) for differentiating a benign adnexal mass from ovarian cancer may be characterized by a specificity of at least 90% (including, *e.g.*, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or higher) and a sensitivity of at least 65% (including, *e.g.*, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or higher). In some embodiments, a particularly useful ovarian cancer screening test (*e.g.*, in some embodiments comprising one or more biomarker combinations described herein, *e.g.*, as shown in **Table 8**) for differentiating a benign adnexal mass from ovarian cancer may be characterized by a specificity of about 90% to about 100% and a sensitivity of about 60% to about 100%, or about 65% to about 95%, or about 70% to about 95%.

[0334] In some embodiments, the present disclosure provides an insight that an ovarian cancer screening test involving more than one set of biomarker combinations (*e.g.*, at least two orthogonal biomarker combinations as described herein) can increase sensitivity of such an assay, as compared to that is achieved by one set of biomarker combination. For example, in some embodiments, an ovarian cancer screening test involving at least two orthogonal biomarker combinations can achieve a specificity of at least 98% and a sensitivity of at least 50%. In some embodiments, an ovarian cancer screening test involving at least two orthogonal biomarker combinations can achieve a specificity of at least 98% and a sensitivity of at least 60%. In some embodiments, an ovarian cancer screening test involving at least two orthogonal biomarker combinations can achieve a specificity of at least 99% and a sensitivity of at least 70%.

[0335] In some embodiments, the present disclosure provides an insight that a particularly useful ovarian cancer screening test may be characterized by an acceptable positive predictive value (PPV) at an economically justifiable cost. PPV is the likelihood a patient has the disease following a positive test, and is influenced by sensitivity, specificity, and/or disease prevalence. One clinician

consensus for the minimum PPV needed to screen for ovarian cancer is 10% (Nossov *et al.*, 2008; which is incorporated herein by reference for the purpose described herein). With a 10% PPV, there would be nine false positives for every one true positive. These false positives place a significant burden on both the healthcare system and the women being screened as they lead to additional tests, unnecessary surgeries, and emotional and physical distress (Buys *et al.*, 2011; which is incorporated herein by reference for the purpose described herein). In some embodiments, assays described herein are particularly useful for early ovarian cancer detection that achieves a PPV of greater than 10% or higher, including, *e.g.*, greater than 15%, greater than 20%, or greater than 25% or higher, with a specificity cutoff of at least 98% for women at hereditary risk for ovarian cancer, or with a specificity cutoff of at least 99.5% for women experiencing one or more symptoms associated with ovarian cancer.

[0336] In some embodiments, assays described herein can be useful for early ovarian cancer detection that achieves a PPV of greater than 2% or higher, including, *e.g.*, greater than 3%, greater than 4%, greater than 5%, greater than 6%, greater than 7%, greater than 8%, greater than 9%, greater than 10%, greater than 15%, greater than 20%, or greater than 25% or higher. In some such embodiments, assays described herein can achieve a specificity cutoff of at least 95% or higher (*e.g.*, a specificity cutoff of at least 98% for women at hereditary risk for ovarian cancer, or with a specificity cutoff of at least 99.5% for women experiencing one or more symptoms associated with ovarian cancer).

[0337] In some embodiments, assays described herein (*e.g.*, in some embodiments comprising one or more biomarker combinations described herein, *e.g.*, as shown in **Table 8**) can be useful for differentiating a benign adnexal mass from ovarian cancer that achieves a positive predictive value (PPV) of greater than 65% or higher (including, *e.g.*, greater than 70%, greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 98%, greater than 99%, or higher), and/or a negative predictive value (NPV) of greater than 90% (including, *e.g.*, greater than 95%, greater than 96%, greater than 97%, greater than 98%, or higher).

[0338] Several different biomarker classes have been studied for an ovarian cancer liquid biopsy assay including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), bulk proteins, and extracellular vesicles (EVs). EVs are particularly promising due to their abundance and stability in the bloodstream relative to ctDNA and CTCs, suggesting improved sensitivity for early-stage cancers. Moreover, EVs contain cargo (*i.e.*, proteins, RNA, metabolites) that originated from the same cell, providing superior specificity over bulk protein measurements. While the diagnostic utility EVs has been studied, much of this work has pertained to bulk EV measurements or low-throughput single-EV analyses.

II. Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer

[0339] The present disclosure, among other things, provides various target biomarkers or combinations thereof (*e.g.*, target biomarker signatures) for ovarian cancer. Such target biomarker signatures that are predicted to exhibit high sensitivity and specificity for ovarian cancer were discovered by a multi-pronged bioinformatics analysis and biological approach, which for example, in some embodiments involve computational analysis of a diverse set of data, *e.g.*, in some embodiments comprising one or more of sequencing data, expression data, mass spectrometry, histology, post-translational modification data, and/or *in vitro* and/or *in vivo* experimental data through machine learning and/or computational modeling. In some embodiments, biomarker combinations described herein have been demonstrated to achieve at least 99% specificity with certain sensitivity (*e.g.*, in some embodiments at least 70% sensitivity) when they are used to distinguish ovarian cancer samples from reference samples (*e.g.*, normal healthy samples and/or benign tumor samples).

[0340] In some embodiments, a target biomarker signature of ovarian cancer comprises at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or more) surface biomarkers (*e.g.*, in some embodiments surface polypeptide present in extracellular vesicles associated with ovarian cancer; “extracellular vesicle-associated surface biomarker”) and at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or more) target biomarkers comprising one or more surface protein biomarker(s), such that the combination of such surface biomarker(s) and such target biomarker(s) present a target biomarker signature of ovarian cancer that provides (a) high specificity (*e.g.*, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or higher such as at least 99%, or at least 99.5%) to minimize the number of false positives, and (b) high sensitivity (*e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In some embodiments, such target biomarker signatures described herein provides a specificity within a range of 90% to 100% and a sensitivity within a range of 65% to 100%. In some embodiments, such target biomarker signatures described herein provides a specificity within a range of 90% to 100% and a sensitivity within a range of 70% to 95%. In some embodiments, such target biomarker signatures described herein are particularly useful for detection of stage I and II ovarian cancer when prognosis is most favorable. In some embodiments, such target biomarker signatures described herein are particularly useful for differentiating a benign adnexal mass from ovarian cancer.

[0341] In some embodiments, a target biomarker signature of ovarian cancer comprises at least one surface biomarker (*e.g.*, surface polypeptide and/or carbohydrate-dependent marker present on the surfaces of extracellular vesicles associated with ovarian cancer) and at least one target biomarker comprising one or more surface protein biomarker(s), such that the combination of such surface biomarker(s) and such target biomarker(s) present a target biomarker signature of ovarian cancer that provides a positive predictive value (PPV) at least 15% or higher, at least 20% or higher,

at least 25% or higher, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or higher. In some embodiments, a target biomarker signature of ovarian cancer comprises at least one surface biomarker (*e.g.*, surface polypeptide and/or carbohydrate-dependent marker present on the surfaces of extracellular vesicles associated with ovarian cancer) and at least one target biomarker comprising one or more surface protein biomarker(s), such that the combination of such surface biomarker(s) and such target biomarker(s) present a target biomarker signature of ovarian cancer that provides a positive predictive value (PPV) of greater than 2% or higher, including, *e.g.*, greater than 3%, greater than 5%, greater than 7%, greater than 10%, greater than 15% or higher, greater than 20% or higher, greater than 25% or higher, and/or greater than 30% or higher. In some embodiments, such a target biomarker signature described herein provides a PPV within a range of 70% to 100% or within a range of 70% to 90%.

[0342] In some embodiments, the present disclosure recognizes that in certain embodiments, sensitivity and specificity rates for women with different ovarian risk levels may vary depending upon the risk tolerance of the attending physician and/or the guidelines set forth by interested medical consortia. In some embodiments, lower specificity and/or sensitivity may be used for screening patients at higher risk of ovarian cancer (*e.g.*, patients with life-history-associated risk factors, symptomatic patients, or patients with a family history of ovarian cancer, *etc.*) as compared to that for patients with lower risk for ovarian cancer. For example, in some embodiments, biomarker combinations described herein that are useful for detection of ovarian cancer may provide a specificity of at least 70% including, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99.5%, or higher. Additionally or alternatively, in some embodiments, biomarker combinations described herein that are useful for detection of ovarian cancer may provide a sensitivity of at least 50% including, *e.g.*, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99.5%, or higher.

[0343] In certain embodiments, women with hereditary risk of ovarian cancer may be best served with a 99.5% specificity rate with 70% sensitivity or a 98% specificity rate with 80% sensitivity or a 99% specificity rate with 70% sensitivity. In certain embodiments, post-menopausal non-symptomatic women may be best served with a 99.5% specificity rate with 70% sensitivity or a 98% specificity rate with 80% sensitivity or a 99% specificity rate with 70% sensitivity. In certain embodiments, post-menopausal symptomatic women may be best served with a 99.5% specificity rate with 70% sensitivity or a 98% specificity rate with 80% sensitivity or a 99% specificity rate with 70% sensitivity. In certain embodiments, women with life-history risk may be best served with a 99.5% specificity rate with 70% sensitivity or a 98% specificity rate with 80% sensitivity or a 99% specificity rate with 70% sensitivity. In some embodiments, technologies and/or assays described herein for detection of ovarian cancer in a symptomatic woman may have a lower sensitivity and/or

specificity requirement than those for detection of ovarian cancer in an asymptomatic woman. In some embodiments, an assay described herein for detection of ovarian cancer in a symptomatic woman may have a set specificity rate that is lower than 99.5% specificity, including *e.g.*, less than 99% sensitivity, less than 95%, less than 90%, or less than 85% specificity rate. In some embodiments, an assay described herein for detection of ovarian cancer in a symptomatic woman may have a set sensitivity rate that is lower than 80% sensitivity, including *e.g.*, less than 70%, or less than 60% sensitivity rate.

[0344] In general, gene identifiers used herein refer to the Gene Identification catalogued by the UniProt Consortium (UniProt.org); one skilled in the art will understand that certain genes can be known by multiple names and will also readily recognize such multiple names.

[0345] In general, carbohydrate identifiers used herein refer to Kegg Cancer-associated Carbohydrates database (genome.jp/kegg/disease/br08441.html); one skilled in the art will understand that certain carbohydrates can be known by multiple names and will also readily recognize such multiple names.

[0346] In certain embodiments, a target biomarker signature of ovarian cancer comprises at least one extracellular vesicle-associated surface biomarker (*e.g.*, surface polypeptide and/or carbohydrate-dependent marker present in nanoparticles associated with ovarian cancer) and at least one target biomarker comprising one or more surface protein biomarker(s), such that the combination of such extracellular vesicle-associated surface biomarker(s) and such target biomarker(s) is specific for ovarian cancer. In some embodiments, extracellular vesicle-associated surface biomarkers and/or target surface biomarkers may be selected from: basal cell adhesion molecule polypeptide encoded by the basal cell adhesion molecule (BCAM) gene, bone marrow stromal cell antigen 2 polypeptide encoded by the bone marrow stromal cell antigen 2 (BST2) gene, claudin 3 polypeptide encoded by the claudin 3 (CLDN3) gene, cleaved mucin 16 polypeptide partially encoded by the mucin 16, cell surface associated (MUC16) gene, folate receptor alpha polypeptide encoded by the folate receptor alpha (FOLR1) gene, mesothelin polypeptide encoded by the mesothelin (MSLN) gene, mucin 1 polypeptide encoded by the mucin 1, cell surface associated (MUC1) gene, mucin 16 polypeptide encoded by the mucin 16, cell surface associated (MUC16) gene, sodium-dependent phosphate transport protein 2B polypeptide encoded by the solute carrier family 34 (sodium phosphate) member 2 (SLC34A2) gene, SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0347] In some embodiments, extracellular vesicle-associated surface biomarkers and/or target surface biomarkers may be selected from: bone marrow stromal cell antigen 2 polypeptide encoded by the bone marrow stromal cell antigen 2 (BST2) gene, folate receptor alpha polypeptide encoded by the folate receptor alpha (FOLR1) gene, mesothelin polypeptide encoded by the

mesothelin (MSLN) gene, mucin 1 polypeptide encoded by the mucin 1, cell surface associated (MUC1) gene, mucin 16 polypeptide encoded by the mucin 16, cell surface associated (MUC16) gene, SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof. In some embodiments, a mucin 16 polypeptide is an intact polypeptide. In some embodiments, a mucin 16 polypeptide is a cleaved polypeptide.

[0348] In certain embodiments, a target biomarker signature of ovarian cancer is or comprises one or more (*e.g.*, at least one, at least two, at least three, or more) of the following surface biomarkers: basal cell adhesion molecule (BCAM) polypeptide, bone marrow stromal cell antigen 2 (BST2) polypeptide, claudin-3 (CLDN3) polypeptide, cleaved mucin-16 (cleaved MUC16) polypeptide, folate receptor alpha (FOLR1) polypeptide, mesothelin (MSLN) polypeptide, mucin-1 (MUC1) polypeptide, mucin-16 (MUC16) polypeptide, sodium-dependent phosphate transport protein 2B (SLC34A2) polypeptide, SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0349] In certain embodiments, a target biomarker signature of ovarian cancer is or comprises one or more (*e.g.*, at least one, at least two, at least three, or more) of the following surface biomarkers: bone marrow stromal cell antigen 2 (BST2) polypeptide, folate receptor alpha (FOLR1) polypeptide, mesothelin (MSLN) polypeptide, mucin-1 (MUC1) polypeptide, mucin-16 (MUC16) polypeptide, SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof. In some embodiments, a mucin 16 polypeptide is a cleaved polypeptide.

[0350] In certain embodiments, a target biomarker signature for ovarian cancer detection (*e.g.*, HGSOc) comprises a combination of at least two target surface biomarkers, which combination can be selected from the following: a MUC16 polypeptide and a FOLR1 polypeptide; or a SLC34A2 polypeptide and a FOLR1 polypeptide; or a SLC34A2 polypeptide and a MUC16 polypeptide; or a BST2 polypeptide and a FOLR1 polypeptide; or a CA19-9 antigen and a BST2 polypeptide; or a CA19-9 antigen and a CLDN3 polypeptide; or a CA19-9 antigen and a SLC34A2 polypeptide; or a MUC1 polypeptide and a BCAM polypeptide; or a MUC1 polypeptide and a BST2 polypeptide; or a MUC1 polypeptide and a MSLN polypeptide; or a MUC1 polypeptide and a sTn antigen; or a MUC16 polypeptide and a BCAM polypeptide; or a MUC16 polypeptide and a MUC1 polypeptide; or a MUC16 polypeptide and a sTn antigen; or a sTn antigen and a FOLR1 polypeptide; or a T antigen and BST2 polypeptide; or combinations thereof. In certain embodiments, a target biomarker described in the foregoing combinations may be used as a target of a capture probe and/or a target of a detection probe of assays described herein.

[0351] In certain embodiments, a target biomarker signature for ovarian cancer detection (*e.g.*, HGSOc) comprises a combination of at least three target surface biomarkers, which combination can be selected from the following: a CA19-9 antigen and a BST2 polypeptide and a MUC16 polypeptide; a MUC1 polypeptide and a BCAM polypeptide and a BST2 polypeptide; or a

MUC1 polypeptide and a BST2 polypeptide and a FOLR1 polypeptide; or a MUC1 polypeptide and a BST2 polypeptide and a sTn antigen; or a MUC1 polypeptide and a MSLN polypeptide and a sTn antigen; or a MUC16 polypeptide and a FOLR1 polypeptide and a SLC34A2 polypeptide; or a MUC16 polypeptide and a MUC1 polypeptide and a sTn antigen; or a MUC16 polypeptide and a MSLN polypeptide and a sTn antigen; or a MUC16 polypeptide and a SLC34A2 polypeptide and a sTn antigen; or a sTn antigen and a FOLR1 polypeptide and a MUC16 polypeptide; or a sTn antigen and a FOLR1 polypeptide and MSLN polypeptide; or a sTn antigen and a FOLR1 polypeptide and a MUC1 polypeptide; or a sTn antigen and a MUC1 antigen and a SLC34A2 antigen; or a sTn antigen and a MUC16 polypeptide and a cleaved MUC 16 polypeptide; or a sTn antigen and a cleaved MUC16 polypeptide and a MSLN polypeptide; or a sTn antigen and a FOLR1 polypeptide and a SLC34A2 polypeptide; or combinations thereof. In certain embodiments, a target biomarker described in the foregoing combinations may be used as a target of a capture probe and/or a target of a detection probe of assays described herein.

[0352] In some embodiments, a target biomarker signature may comprise targets of a combination as depicted in Table 1, wherein a target may be used in a capture probe and/or detection probe. In some embodiments, a target biomarker signature may comprise a target of capture probe as depicted in Table 1 and at least one or more (including, *e.g.*, at least two or more) targets of detection probes (*e.g.*, detection probe 1 and/or detection probe 2). By way of example only, in some embodiments, a target biomarker signature may comprise MUC16 (a target of capture probe depicted in Table 1), sTn antigen (a target of detection probe 1 or 2 depicted in Table 1) and FOLR1 (a target of detection probe 1 or 2 depicted in Table 1). In some embodiments, a target biomarker signature may comprise targets of a combination of capture and detection probes as depicted in *Table 1*. A skilled artisan reading the present disclosure will understand that targets of “Detection Probe 1” and “Detection Probe 2” in a given combination can be used interchangeably.

Table 1 exemplary target biomarker signature probe combinations.

Combination	Target of Capture Probe	Target of Detection Probe 1	Target of Detection Probe 2
1	MUC16	FOLR1	MUC16
2	SLC34A2	FOLR1	FOLR1
3	SLC34A2	MUC16	MUC16
4	BST2	FOLR1	FOLR1
5	CA19-9	BST2	BST2
6	CA19-9	BST2	MUC16
7	CA19-9	CLDN3	CLDN3
8	CA19-9	SLC34A2	SLC34A2
9	MUC1	BCAM	BCAM
10	MUC1	BCAM	BST2
11	MUC1	BST2	BST2
12	MUC1	BST2	FOLR1
13	MUC1	BST2	MUC1
14	MUC1	BST2	sTn

15	MUC1	MSLN	MUC1
16	MUC1	MSLN	sTn
17	MUC1	sTn	sTn
18	MUC16	BCAM	BCAM
19	MUC16	FOLR1	FOLR1
20	MUC16	FOLR1	SLC34A2
21	MUC16	MUC1	MUC1
22	MUC16	MUC1	MUC16
23	MUC16	MUC1	sTn
24	MUC16	MUC16	sTn
25	MUC16	MSLN	sTn
26	MUC16	SLC34A2	SLC34A2
27	MUC16	SLC34A2	sTn
28	MUC16	sTn	sTn
29	sTn	BST2	MUC1
30	sTn	FOLR1	MUC1
31	sTn	FOLR1	MUC16
32	sTn	FOLR1	MSLN
33	sTn	FOLR1	FOLR1
34	sTn	MUC1	MSLN
35	sTn	MUC1	SLC34A2
36	sTn	MUC16	cleaved MUC16
37	sTn	MUC16	MSLN
38	sTn	cleaved MUC16	MSLN
39	sTn	FOLR1	SLC34A2
40	T	BST2	BST2

[0353] In some embodiments, certain biomarker combinations as depicted in Table 1 that may be particularly useful (*e.g.*, with higher sensitivity, specificity and/or PPV) for ovarian cancer detection can undergo one or more rounds of screening using an advanced stage (*e.g.*, late stage, *e.g.*, stage III and/or IV) ovarian cancer samples (*e.g.*, pooled or individual samples) and the healthy control samples (*e.g.*, pooled or individual samples) as a reference. In some embodiments, select combinations can be further tested using early-stage ovarian cancer samples (*e.g.*, stage I and/or II, optionally differentiated by low or high CA-125 content), benign gynecological tumor plasma samples (*e.g.*, as described herein), non-ovarian cancer samples (*e.g.*, as described herein), and/or any combination thereof. In some embodiments, biomarker combination performance can be determined by calculating the difference in assay signal (*e.g.*, on a Ct basis) between the healthy samples (*e.g.*, pooled samples and/or individual samples) and ovarian cancer samples (*e.g.*, pooled samples and/or individual samples).

[0354] In some embodiments, certain biomarker combinations for ovarian cancer detection can be selected with a delta Ct greater than inter-assay variability. For example, in some embodiments, biomarker combinations with a delta Ct greater than 2.0 (corresponding to a fourfold difference) or 1.0 (corresponding to a twofold difference) are considered to provide particularly

effective diagnostic utility (*e.g.*, providing a signal greater than inter-assay variability). See, *e.g.*, *Examples 2-3*, which provide exemplary analyses of certain combinations described herein.

[0355] In certain embodiments, a target biomarker signature for ovarian cancer is one that differentiates late-stage ovarian cancer samples from a control sample (*e.g.*, compared to healthy samples, compared to benign gynecological tumor samples, and/or compared to other cancer samples). In certain embodiments, a target biomarker signature for ovarian cancer is one that differentiates early stage ovarian cancer samples (*e.g.*, with low and/or high plasma CA-125) from a control sample (*e.g.*, compared to healthy samples, compared to benign gynecological tumor samples, and/or compared to other cancer samples). In some embodiments, an assay directed to detection of a target biomarker signature for ovarian cancer can comprise a combination of capture and detection probes as described in *Table 1*.

[0356] In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to FOLR1 and MUC16, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC16 and MUC16, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and BST2, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and MUC16, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to CLDN3 and CLDN3, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to SLC34A2 and SLC34A2, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BCAM and BCAM, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BCAM and BST2, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and FOLR1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and MUC1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MSLN and MUC1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to sTn antigen and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to FOLR1 and SLC34A2, respectively. In certain

embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC1 and MUC1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC1 and MUC16, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC1 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC16 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to SLC34A2 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to FOLR1 and MSLN, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC16 and MSLN, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to FOLR1 and MUC1, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC1 and SLC34A2, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC16 and cleaved MUC16, respectively. In certain embodiments, at least two detection probes in a plurality may have their target binding entities directed to cleaved MUC16 and MSLN, respectively.

[0357] In certain embodiments, wherein a target biomarker signature comprises a combination of SLC34A2 and FOLR1, a capture probe has their target binding entity directed to SLC34A2 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of SLC34A2 and MUC16, a capture probe has their target binding entity directed to SLC34A2 and at least two detection probes have their target binding entities directed to MUC16 and MUC16, respectively.

[0358] In certain embodiments, wherein a target biomarker signature comprises a combination of BST2 and FOLR1, a capture probe has their target binding entity directed to BST2 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively.

[0359] In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and BST2, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and BST2 and MUC16, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to BST2 and MUC16, respectively. In certain embodiments, wherein a target biomarker

signature comprises a combination of CA19-9 antigen and CLDN3, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to CLDN3 and CLDN3, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and SLC34A2, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to SLC34A2 and SLC34A2, respectively.

[0360] In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BCAM, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BCAM and BCAM, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BCAM and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BCAM and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2 and FOLR1, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2 and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and MSLN, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to MSLN and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and MSLN and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to sTn antigen and sTn antigen, respectively.

[0361] In certain embodiments wherein a target biomarker signature comprises a combination of MUC16 and FOLR1, a capture probe has their target binding entity directed to

MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and BCAM, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to BCAM and BCAM, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and FOLR1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and FOLR1 and SLC34A2, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC16 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MSLN and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and SLC34A2, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to SLC34A2 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and SLC34A2 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to SLC34A2 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to sTn antigen and sTn antigen, respectively.

[0362] In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and BST2 and MUC1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to BST2 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MUC16, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC1 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC1 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC16 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC16 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MUC1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC1 and SLC34A2, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC1 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC16 and cleaved MUC16, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC16 and cleaved MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and cleaved MUC16 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to cleaved MUC16 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and SLC34A2, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and SLC34A2, respectively.

[0363] In certain embodiments, wherein a target biomarker signature comprises a combination of T antigen and BST2, a capture probe has their target binding entity directed to T antigen and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively.

[0364] In some embodiments, a target biomarker signature for ovarian cancer comprises at least two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) surface biomarkers (e.g., ones described herein) present on the surface of nanoparticles having a size range of interest that includes extracellular vesicles, e.g., in some embodiments, nanoparticles having a size within the range of about 30 nm to about 1000 nm.) In some embodiments, the two or more surface biomarkers are the same. In some embodiments, the two or more surface biomarkers are distinct.

[0365] In some embodiments, a target biomarker signature for ovarian cancer comprises at least one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, or more) extracellular vesicle-associated surface biomarkers (e.g., ones described herein) and at least one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, or more) surface biomarkers (e.g., ones described herein). In some embodiments, at least one extracellular vesicle-associated surface biomarker and at least one surface biomarker are the same.

[0366] In some embodiments, at least one extracellular vesicle-associated surface biomarker and at least one surface biomarker(s) of a target biomarker signature for ovarian cancer are distinct. For example, in some embodiments, a target biomarker signature for ovarian cancer comprises at least one extracellular vesicle-associated surface biomarker and at least one surface biomarker.

[0367] In some embodiments, a target biomarker signature comprises at least one of the following biomarker combinations:

- i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

In some embodiments, such a target biomarker signature is particularly useful for detection of early-stage cancer. In some embodiments, such a target biomarker signature is particularly useful for differentiating a benign adnexal mass from ovarian cancer.

[0368] In some embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) target biomarker signatures described herein can be used in a set for detection of ovarian cancer. For example, in some embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7) of the following target biomarker signatures can be used in a set for detection of ovarian cancer:

- i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

In some embodiments, such a set can be particularly useful for detection of early-stage ovarian cancer. In some embodiments, such a set can be particularly useful for differentiating a benign adnexal mass from ovarian cancer.

[0369] In some embodiments, a target biomarker signature for ovarian cancer (*e.g.*, ones described herein) can further comprise at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or more) intravesicular biomarkers (*e.g.*, ones described herein). In some such embodiments, at least two of the extracellular vesicle-associated surface biomarker(s), the target surface biomarker(s), and the intravesicular biomarker(s) can be encoded by the same gene, while the former is expressed in on surface of extracellular vesicle and the latter is expressed within the extracellular vesicle. In some embodiments, extracellular vesicle-associated surface biomarker(s), target surface biomarker(s) and the intravesicular biomarker(s) can be encoded by different genes. Non-limiting examples of intravesicular biomarkers (*e.g.*, intravesicular protein biomarkers) include CRABP2, KLK7, MIF, PRAME, S100A1, or combinations thereof.

[0370] In some embodiments, a target biomarker signature for ovarian cancer (*e.g.*, ones described herein) can further comprise at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or more) intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding

RNA, or piwi-interacting RNA) biomarkers (*e.g.*, ones described herein). In some such embodiments, at least two of the extracellular vesicle-associated surface biomarker(s), the target surface biomarker(s), and the intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) biomarker(s) can be encoded by the same gene, while the former is expressed in on surface of extracellular vesicle and the latter is expressed within the extracellular vesicle. In some embodiments, extracellular vesicle-associated surface biomarker(s), target surface biomarker(s) and the intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) biomarker(s) can be encoded by different genes. Non-limiting examples of intravesicular RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) biomarkers include CLDN6, CRABP2, KLK7, MIF, PRAME, S100A1, or combinations thereof.

[0371] In some embodiments, any one of the provided biomarkers can be detected and/or measured by protein and/or RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) expression levels in wild-type form.

[0372] In some embodiments, any one of the provided biomarkers can be detected and/or measured by protein and/or RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) expression levels in mutant form. Thus, in some embodiments, mutant-specific detection of provided biomarkers (*e.g.*, proteins and/or RNA such as, *e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) can be included.

[0373] As noted herein, in some embodiments, a biomarker is or comprises a particular form of one or more polypeptides or proteins (*e.g.*, a pro-form, a truncated form, a modified form such as a glycosylated, phosphorylated, acetylated, methylated, ubiquitylated, or lipidated form). In some embodiments, detection of such form detects a plurality (and, in some embodiments, substantially all) polypeptides present in that form (*e.g.*, containing a particular modification such as, for example, a particular glycosylation, *e.g.*, sialyl-Tn (sTn) glycosylation, *e.g.*, a truncated *O*-glycan containing a sialic acid α -2,6 linked to GalNAc α -O-Ser/Thr).

[0374] Accordingly, in some embodiments, a surface biomarker can be or comprise a glycosylation moiety (*e.g.*, an sTn antigen moiety, a Tn antigen moiety, or a T antigen moiety). Thompsen-nouvelle (Tn) antigen is an O-linked glycan that is thought to be associated with a broad array of tumors. Tn is a single alpha-linked GalNAc added to Ser or Thr as the first step of a major O-linked glycosylation pathway. A skilled artisan will understand that in certain embodiments, T antigen typically refers to an O-linked glycan with the structure Gal β 1-3GalNAc-.

[0375] In some embodiments, a surface protein biomarker can be or comprise a tumor-associated post-translational modification. In some embodiments, such a post-translational

modification can be or comprise tumor-specific glycosylation patterns such as mucins with glycans aberrantly truncated at the initial GalNAc (*e.g.*, Tn), or combinations thereof. In some embodiments, a surface protein biomarker can be or comprise a tumor-specific proteoform of mucin resulting from altered splicing and/or translation (isoforms) or proteolysis (cancer specific protease activity resulting in aberrant cleavage products).

[0376] In some embodiments, a biomarker is or comprises a cleaved form of a polypeptide. For example, in some embodiments, a MUC16 biomarker is a cleaved form of a MUC16 protein.

[0377] In some embodiments, an ovarian cancer detection assay described herein can utilize one or more (*e.g.*, at least 1, at least 2, at least 3, or more) biomarker combinations or target biomarker signatures described herein. In some embodiments, an ovarian cancer detection assay described herein can utilize one or more (*e.g.*, at least 1, at least 2, at least 3, or more) biomarker combinations or target biomarker signatures described herein and one or more (*e.g.*, at least 1, at least 2, at least 3, or more) biomarker combinations described in WO 2021/146659, the entire contents of which are incorporated by reference for purposes described herein.

III. Exemplary Methods of Detecting Provided Markers and/or Target Biomarker Signatures for Ovarian Cancer

[0378] In general, the present disclosure provides technologies according to which a target biomarker signature is analyzed and/or assessed in a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) comprising nanoparticles (including, *e.g.*, extracellular vesicles) from a subject in need thereof; in some embodiments, a diagnosis or therapeutic decision is made based on such analysis and/or assessment.

[0379] In some embodiments, methods of detecting a target biomarker signature include methods for detecting one or more provided markers of a target biomarker signature as proteins, glycans, or proteoglycans (including, *e.g.*, but not limited to a protein with a carbohydrate or glycan moiety). Exemplary protein-based methods of detecting one or more provided markers include, but are not limited to, proximity ligation assay, mass spectrometry (MS) and immunoassays, such as immunoprecipitation; Western blot; ELISA; immunohistochemistry; immunocytochemistry; flow cytometry; and immuno-PCR. In some embodiments, an immunoassay can be a chemiluminescent immunoassay. In some embodiments, an immunoassay can be a high-throughput and/or automated immunoassay platform.

[0380] In some embodiments, methods of detecting one or more provided markers as proteins, glycans, or proteoglycans (including, *e.g.*, but not limited to a protein with a carbohydrate or glycan moiety) in a sample comprise contacting a sample with one or more antibody agents directed to the provided markers of interest. In some embodiments, such methods also comprise

contacting the sample with one or more detection labels. In some embodiments, antibody agents are labeled with one or more detection labels.

[0381] In some embodiments, detecting binding between a biomarker of interest and an antibody agent for the biomarker of interest includes determining absorbance values or emission values for one or more detection agents. For example, the absorbance values or emission values are indicative of amount and/or concentration of biomarker of interest expressed by nanoparticles (*e.g.*, higher absorbance is indicative of higher level of biomarker of interest expressed by nanoparticles). In some embodiments, absorbance values or emission values for detection agents are above a threshold value. In some embodiments, absorbance values or emission values for detection agents is at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2.0, at least 2.5, at least 3.0, at least 3.5-fold or greater than a threshold value. In some embodiments, the threshold value is determined across a population of a control or reference group (*e.g.*, non-cancer subjects).

[0382] In some embodiments, methods of detecting one or more provided markers include methods for detecting one or more provided markers as nucleic acids. Exemplary nucleic acid-based methods of detecting one or more provided markers include, but are not limited to, performing nucleic acid amplification methods, such as polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA), ligase chain reaction (LCR), strand displacement amplification (SDA), and nucleic acid sequence-based amplification (NASBA). In some embodiments, a nucleic acid-based method of detecting one or more provided markers includes detecting hybridization between one or more nucleic acid probes and one or more nucleotide sequences that encode a biomarker of interest. In some embodiments, the nucleic acid probes are each complementary to at least a portion of one of the one or more nucleotide sequences that encode the biomarker of interest. In some embodiments, the nucleotide sequences that encode the biomarker of interest include DNA (*e.g.*, cDNA). In some embodiments, the nucleotide sequences that encode the biomarker of interest include RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA).

[0383] In some embodiments, methods of detecting one or more provided markers involve proximity-ligation-immuno quantitative polymerase chain reaction (pliq-PCR). Pliq-PCR can have certain advantages over other technologies to profile EVs. For example, pliq-PCR can have a sensitivity three orders of magnitude greater than other standard immunoassays, such as ELISAs (Darmanis *et al.*, 2010; which is incorporated herein by reference for the purpose described herein). In some embodiments, a pliq-PCR reaction can be designed to have an ultra-low LOD, which enables to detect trace levels of tumor-derived EVs, for example, down to a thousand EVs per mL.

[0384] In some embodiments, methods for detecting one or more provided markers may involve other technologies for detecting EVs, including, *e.g.*, Nanoplasmonic Exosome (nPLEX)

Sensor (Im *et al.*, 2014; which is incorporated herein by reference for the purpose described herein) and the Integrated Magnetic-Electrochemical Exosome (iMEX) Sensor (Jeong *et al.*, 2016; which is incorporated herein by reference for the purpose described herein), which have reported LODs of $\sim 10^3$ and $\sim 10^4$ EVs, respectively (Shao *et al.*, 2018; which is incorporated herein by reference for the purpose described herein).

[0385] In some embodiments, methods for detecting one or more provided biomarkers in nanoparticles can be based on bulk EV sample analysis.

[0386] In some embodiments, methods for detecting one or more provided biomarkers in nanoparticles can be based on profiling individual EVs (*e.g.*, single-EV profiling assays), which is further discussed in the section entitled “*Exemplary Methods for Profiling Individual Nanoparticles*” below.

[0387] A skilled artisan reading the present disclosure will understand that the assays described herein for detecting or profiling individual EVs can be also used to detect biomarker combinations on the surface of nanoparticles as described herein.

[0388] In some embodiments, nanoparticles in a sample may be captured or immobilized on a solid substrate prior to detecting one or more provided biomarkers in accordance with the present disclosure. In some embodiments, nanoparticles may be captured on a solid substrate surface by non-specific interaction, including, *e.g.*, adsorption. In some embodiments, nanoparticles may be selectively captured on a solid substrate surface. For example, in some embodiments, a solid substrate surface may be coated with an agent that specifically binds to nanoparticles (*e.g.*, an antibody agent specifically targeting such nanoparticles, *e.g.*, associated with ovarian cancer). In some embodiments, a solid substrate surface may be coated with a member of an affinity binding pair and an entity of interest (*e.g.*, nanoparticles including, *e.g.*, extracellular vesicles) to be captured may be conjugated to a complementary member of the affinity binding pair. In some embodiments, an exemplary affinity binding pair includes, *e.g.*, but is not limited to biotin and avidin-like molecules such as streptavidin. As will be understood by those of skilled in the art, other appropriate affinity binding pairs can also be used to facilitate capture of an entity of interest to a solid substrate surface. In some embodiments, an entity of interest may be captured on a solid substrate surface by application of a current, *e.g.*, as described in Ibsen *et al.* ACS Nano., 11: 6641-6651 (2017) and Lewis *et al.* ACS Nano., 12: 3311-3320 (2018), both of which are incorporated herein by reference for the purpose described herein, and both of which describe use of an alternating current electrokinetic microarray chip device to isolate extracellular vesicles from an undiluted human blood or plasma sample.

[0389] A solid substrate may be provided in a form that is suitable for capturing nanoparticles and does not interfere with downstream handling, processing, and/or detection. For example, in some embodiments, a solid substrate may be or comprise a bead (*e.g.*, a magnetic bead).

In some embodiments, a solid substrate may be or comprise a surface. For example, in some embodiments, such a surface may be a capture surface of an assay chamber (including, *e.g.*, a tube, a well, a microwell, a plate, a filter, a membrane, a matrix, *etc.*). Accordingly, in some embodiments, a method described herein comprises, prior to detecting provided biomarkers in a sample, capturing or immobilizing nanoparticles on a solid substrate.

[0390] In some embodiments, a sample may be processed, *e.g.*, to remove undesirable entities such as cell debris or cells, prior to capturing nanoparticles on a solid substrate surface. For example, in some embodiments, such a sample may be subjected to centrifugation, *e.g.*, to remove cell debris, cells, and/or other particulates. Additionally, or alternatively, in some embodiments, such a sample may be subjected to size-exclusion-based purification or filtration. Various size-exclusion-based purification or filtration are known in the art and those skilled in the art will appreciate that in some cases, a sample may be subjected to a spin column purification based on specific molecular weight or particle size cutoff. Those skilled in the art will also appreciate that appropriate molecular weight or particle size cutoff for purification purposes can be selected, *e.g.*, based on the size of extracellular vesicles. For example, in some embodiments, size-exclusion separation methods may be applied to samples comprising nanoparticles to isolate a fraction of nanoparticles that include extracellular vesicles of a certain size (*e.g.*, greater than 30 nm and no more than 1000 nm, or greater than 70 nm and no more than 200 nm). Typically, nanoparticles may range from 30 nm to several micrometers in diameter. See, *e.g.*, Chuo *et al.*, “Imaging extracellular vesicles: current and emerging methods” *Journal of Biomedical Sciences* 25: 91 (2018) which is incorporated herein by reference for the purpose described herein, which provides information of sizes for different extracellular vesicle (EV) subtypes: migrasomes (0.5-3 μm), microvesicles (0.1-1 μm), oncosomes (1-10 μm), exomeres (<50 nm), small exosomes (60-80 nm), and large exosomes (90-120 nm). In some embodiments, nanoparticles having a size range of about 30 nm to 1000 nm may be isolated, for example, in some embodiments by one or more size-exclusion separation methods, for detection assay. In some embodiments, specific EV subtype(s) may be isolated, for example, in some embodiments by one or more size-exclusion separation methods, for detection assay.

[0391] In some embodiments, nanoparticles in a sample may be processed prior to detecting one or more provided biomarkers of a target biomarker signature for ovarian cancer. Different sample processing and/or preparation can be performed, *e.g.*, to stabilize targets (*e.g.*, target biomarkers) in nanoparticles to be detected, and/or to facilitate exposure of targets (*e.g.*, intravesicular proteins and/or RNA such as mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA) to a detection assay (*e.g.*, as described herein), and/or to reduce non-specific binding. Examples of such sample processing and/or preparation are known in the art and include, but are not limited to, crosslinking molecular targets

(*e.g.*, fixation), permeabilization of biological entities (*e.g.*, cells or nanoparticles having a size range of interest that include extracellular vesicles), and/or blocking non-specific binding sites.

[0392] In one aspect, the present disclosure provides a method for detecting whether a target biomarker signature of ovarian cancer is present or absent in a biological sample from a subject in need thereof, which may be in some embodiments a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) comprising nanoparticles. In some embodiments, such a method comprises (a) detecting, in a biological sample such as a blood-derived sample (*e.g.*, a plasma sample) from a subject, biological entities of interest (including, *e.g.*, nanoparticles) expressing a target biomarker signature of ovarian cancer; and (b) comparing sample information indicative of the level of the target biomarker signature-expressing biological entities of interest (*e.g.*, nanoparticles) in the biological sample (*e.g.*, biological sample) to reference information including a reference threshold level. In some embodiments, a reference threshold level corresponds to a level of biological entities of interest (*e.g.*, nanoparticles) that express such a target biomarker signature in comparable samples from a population of reference subjects, *e.g.*, non-cancer subjects. In some embodiments, exemplary non-cancer subjects include healthy female subjects (*e.g.*, healthy female subjects of specified age ranges, such as *e.g.*, below age 55 or above age 55), female subjects with non-ovarian related health diseases, disorders, or conditions (including, *e.g.*, female subjects having non-ovarian cancer such as lung cancer, colorectal cancer, *etc.*, or female subjects having symptoms of inflammatory disorders, such as inflammatory bowel disease), female subjects having benign ovarian tumors (*e.g.*, a benign mass observed in a fallopian tube and/or on an ovary), and combinations thereof.

[0393] In some embodiments, a sample is pre-screened for certain characteristics prior to utilization in an assay as described herein. In some embodiments, a sample meeting certain pre-screening criteria is more suitable for diagnostic applications than a sample failing pre-screening criteria. For example, in some embodiments samples are visually inspected for appearance using known standards, *e.g.*, is the sample normal, hemolyzed (red), icteric (yellow), and/or lipemic (whitish/turbid). In some embodiments, samples can then be rated on a known standard scale (*e.g.*, 1, 2, 3, 4, 5) and the results are recorded. In some embodiments, samples are visually inspected for hemolysis (*e.g.*, heme) and rated on a scale from 1-5, where the visual inspection correlates with a known concentration, *e.g.*, where 1 denotes approximately 0 mg/dL, 2 denotes approximately 50 mg/dL, 3 denotes approximately 150 mg/dL, 4 denotes approximately 250 mg/dL, and 5 denotes approximately 525 mg/dL. In some embodiments, samples are visually inspected icteric levels (*e.g.*, bilirubin) and rated on a scale from 1-5, where the visual inspection correlates with a known concentration, *e.g.*, where 1 denotes approximately 0 mg/dL, 2 denotes approximately 1.7 mg/dL, 3 denotes approximately 6.6 mg/dL, 4 denotes approximately 16 mg/dL, and 5 denotes approximately 30 mg/dL. In some embodiments, samples are visually inspected for turbidity (*e.g.* lipids) and rated

on a scale from 1-5, where the visual inspection correlates with a known concentration, *e.g.*, where 1 denotes approximately 0 mg/dL, 2 denotes approximately 125 mg/dL, 3 denotes approximately 250 mg/dL, 4 denotes approximately 500 mg/dL, and 5 denotes approximately 1000 mg/dL.

[0394] In some embodiments, samples scoring lower than a certain level on one or more metrics, *e.g.*, equal to or lower than a score of 4, may be utilized in an assay as described herein. In some embodiments, samples scoring lower than a certain level on one or more metrics, *e.g.*, equal to or lower than a score of 3, may be utilized in an assay as described herein. In some embodiments, samples scoring lower than a certain level on one or more metrics, *e.g.*, equal to or lower than a score of 2, may be utilized in an assay as described herein. In some embodiments, samples scoring lower than a certain level on all three metrics (*e.g.*, hemolyzed, icteric, and lipemic) *e.g.*, equal to or lower than a score of 2, may be utilized in an assay as described herein. In some embodiments, low visual inspection scores on pre-screening criteria such as hemolysis, bilirubin, and/or lipemia (*e.g.*, equal to or lower than a score of 2) may have no appreciable effect (*e.g.*, not be correlated with) on diagnostic properties (*e.g.*, Ct values) produced in an assay as described herein.

[0395] In some embodiments, a sample is determined to be positive for the presence of a target biomarker signature (*e.g.*, ones described herein) when it shows an elevated level of target biomarker signature-expressing nanoparticles relative to a reference threshold level (*e.g.*, ones described herein). In some embodiments, a sample is determined to be positive for the presence of a target biomarker signature (*e.g.*, as reflected by the level of target biomarker signature-expressing nanoparticles) if its level is at least 30% or higher, including, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or higher, as compared to a reference threshold level. In some embodiments, a sample is determined to be positive for the presence of a target biomarker signature (*e.g.*, as reflected by the level of target biomarker signature-expressing nanoparticles) if its level is at least 2-fold or higher, including, *e.g.*, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 50-fold, at least 100-fold, at least 250-fold, at least 500-fold, at least 750-fold, at least 1000-fold, at least 2500-fold, at least 5000-fold, or higher, as compared to a reference threshold level.

[0396] In some embodiments, a binary classification system may be used to determine whether a sample is positive for the presence of a target biomarker signature. For example, in some embodiments, a sample is determined to be positive for the presence of a target biomarker signature (*e.g.*, as reflected by the level of target biomarker signature-expressing nanoparticles) if its level is at or above a reference threshold level, *e.g.*, a cutoff value. In some embodiments, such a reference threshold level (*e.g.*, a cutoff value) may be determined by selecting a certain number of standard deviations away from an average value obtained from control subjects such that a desired sensitivity and/or specificity of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, such a reference threshold level (*e.g.*, a cutoff value) may be determined by

selecting a certain number of standard deviations away from a maximum assay signal obtained from control subjects such that a desired sensitivity and/or specificity of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, such a reference threshold level (*e.g.*, a cutoff value) may be determined by selecting the less restrictive of either (i) a certain number of standard deviations away from an average value obtained from control subjects, or (ii) a certain number of standard deviations away from a maximum assay signal obtained from control subjects, such that a desired sensitivity and/or specificity of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, control subjects for determination of a reference threshold level (*e.g.*, a cutoff value) may include, but are not limited to healthy subjects, subjects with inflammatory conditions (*e.g.*, Crohn's disease, ulcerative colitis, endometriosis, *etc.*), subjects with benign gynecological tumors, and combinations thereof. In some embodiments, healthy subjects and subjects with inflammatory conditions (*e.g.*, Crohn's disease, ulcerative colitis, endometriosis, *etc.*) are included in determination of a reference threshold level (*e.g.*, a cutoff value). In some embodiments, subjects with benign gynecological tumors are not included in determination of a reference threshold level (*e.g.*, a cutoff value). In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may be determined by selecting at least 1.5 standard deviations (SDs) or higher (including, *e.g.*, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, at least 2.1, at least 2.2, at least 2.3, at least 2.4, at least 2.5, at least 2.6, at least 2.7, at least 2.8, at least 2.9, at least 3, at least 3.1, at least 3.2, at least 3.3, at least 3.4, at least 3.5, at least 3.6 or higher SDs) away from (i) an average value obtained from control subjects, or (ii) a maximum assay signal obtained from control subjects, such that a desired specificity (*e.g.*, at least 95% or higher specificity [including, *e.g.*, at least 96%, at least 97%, at least 98%, at least 99%, or higher specificity] such as in some embodiments at least 99.8% specificity) of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may be determined by selecting at least 2.9 SDs (*e.g.*, at least 2.93 SDs) away from (i) an average value obtained from control subjects, or (ii) a maximum assay signal obtained from control subjects, such that a desired specificity (*e.g.*, at least 99%, or higher specificity) of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may be determined by selecting at least 2.9 SDs (*e.g.*, at least 2.93 SDs) away from the less restrictive of (i) an average value obtained from control subjects, or (ii) a maximum assay signal obtained from control subjects, such that a desired specificity (*e.g.*, at least 99%, or higher specificity) of an ovarian cancer detection assay (*e.g.*, ones described herein) can be achieved. In some embodiments, such a reference threshold level (*e.g.*, a cutoff value) may be determined based on expression level (*e.g.*, transcript level) of a target biomarker in normal healthy tissues *vs.* in ovarian cancer samples such that the specificity and/or sensitivity of interest (*e.g.*, as described herein) can be achieved. In some embodiments, a reference threshold level (*e.g.*, a cutoff

value) may vary dependent on, for example, ovarian cancer stages and/or subtypes and/or patient characteristics, for example, patient age, menopausal status, risks factors for ovarian cancer (*e.g.*, hereditary risk *vs.* average risk, life-history-associated risk factors), symptomatic/asymptomatic status, and combinations thereof.

[0397] In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may be determined based on a log-normal distribution around healthy female subjects (*e.g.*, of specified age ranges), and optionally female subjects with inflammatory conditions (*e.g.*, Crohn's disease, mastitis, atherosclerosis, heart disease, ulcerative colitis, chronic kidney disease, diabetes, inflammatory bowel disease, fatty liver disease, chronic obstructive pulmonary disease, endometriosis, rheumatoid arthritis, obesity, pancreatitis, *etc.*) but are not cancerous, and selection of a level that is necessary to achieve the specificity of interest, *e.g.*, based on prevalence of ovarian cancer or a subtype thereof. In some embodiments, specificity of interest may be at least 70%, including, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.5% or higher.

[0398] The present disclosure, among other things, also provides technologies for determining whether a subject as having or being susceptible to ovarian cancer, for example, from a sample comprising nanoparticles with a size range of interest that includes extracellular vesicles. For example, in some embodiments, when a biological sample (*e.g.*, in some embodiments a bodily fluid-derived sample such as, *e.g.*, but not limited to a blood-derived sample) from a subject in need thereof shows a level of target biomarker signature-expressing nanoparticles that is at or above a reference threshold level, *e.g.*, cutoff value (*e.g.*, as determined in accordance with the present disclosure), then the subject is classified as having or being susceptible to ovarian cancer. In some such embodiments, a reference threshold level (*e.g.*, cutoff value) may be determined based on a log-normal distribution around healthy female subjects (*e.g.*, of specified age ranges), and optionally female subjects with inflammatory conditions (*e.g.*, Crohn's disease, ulcerative colitis, endometriosis, *etc.*) and selection of a level that is necessary to achieve the specificity of interest, *e.g.*, based on prevalence of ovarian cancer or a subtype thereof. In some embodiments, specificity of interest may be at least 70%, including, *e.g.*, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, at least 99.5% or higher.

[0399] In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may be determined based on expression level (*e.g.*, transcript level) of individual target biomarker(s) of a target biomarker signature in normal healthy tissues *vs.* in ovarian cancer samples such that the specificity and/or sensitivity of interest (*e.g.*, as described herein) can be achieved. In some embodiments, a reference threshold level (*e.g.*, a cutoff value) may vary dependent on, for example, ovarian cancer stages and/or subtypes and/or patient characteristics, for example, patient age, menopausal status, risks factors for ovarian cancer (*e.g.*, hereditary risk *vs.* average risk, life-history-associated risk factors), symptomatic/asymptomatic status, and combinations thereof.

[0400] In some embodiments, when a biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) from a subject in need thereof shows a level of biomarker combination that satisfies a reference threshold level, then the subject is classified as having or being susceptible to ovarian cancer. For example, in some embodiments, when a bodily fluid-derived sample (e.g., but not limited to a blood-derived sample) from a subject in need thereof shows an elevated level of target biomarker signature-expressing extracellular vesicles relative to a reference threshold level, then the subject is classified as having or being susceptible to ovarian cancer. In some embodiments, a subject in need thereof is classified as having or being susceptible to ovarian cancer when the subject's biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) shows a level of target biomarker signature-expressing nanoparticles that is at least 30% or higher, including, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or higher, as compared to a reference threshold level. In some embodiments, a subject in need thereof is classified as having or being susceptible to ovarian cancer when the subject's biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) shows a level of target biomarker signature-expressing nanoparticles that is at least 2-fold or higher, including, e.g., at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, at least 100-fold, at least 250-fold, at least 500-fold, at least 750-fold, at least 1000-fold, or higher, as compared to a reference threshold level.

[0401] When a biological sample (e.g., in some embodiments a bodily fluid-derived sample such as, e.g., but not limited to a blood-derived sample) from a subject in need thereof shows a comparable level to a reference threshold level, then the subject is classified as not likely to have or as not likely to be susceptible to ovarian cancer. In some such embodiments, a reference threshold level corresponds to a level of nanoparticles that express a target biomarker signature in comparable samples from a population of reference subjects, e.g., non-cancer subjects. In some embodiments, exemplary non-cancer subjects include healthy female subjects (e.g., healthy female subjects of specified age ranges, such as e.g., below age 55 or above age 55), female subjects with non-ovarian related health diseases, disorders, or conditions (including, e.g., subjects having non-ovarian cancer such as lung cancer, colorectal cancer, *etc.*, or female subjects having symptoms of inflammatory bowel diseases or disorders but not cancerous), female subjects having benign ovarian tumors (e.g., a benign mass observed in a fallopian tube and/or on an ovary), and combinations thereof.

IV. Exemplary Methods for Profiling Individual Nanoparticles

[0402] In some embodiments, assays for profiling individual extracellular vesicles (*e.g.*, single EV profiling assays) can be used to detect one or more provided biomarkers of one or more target biomarker signatures for ovarian cancer. For example, in some embodiments, such an assay may involve (i) a capture assay through targeting one or more provided markers of a target biomarker signature for ovarian cancer and (ii) a detection assay for at least one or more additional provided markers of such a target biomarker signature for ovarian cancer, wherein such a capture assay is performed prior to such a detection assay.

[0403] A skilled artisan reading the present disclosure will understand that assays described herein for detecting or profiling individual extracellular vesicles can also detect surface biomarkers present on the surfaces of nanoparticles having a size of interest (*e.g.*, in some embodiments a size within the range of about 30 nm to about 1000 nm) that includes extracellular vesicles.

[0404] In some embodiments, a capture assay is performed to selectively capture tumor-associated nanoparticles (*e.g.*, ovarian tumor-associated nanoparticles) from a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample) of a subject in need thereof. In some embodiments, a capture assay is performed to selectively capture nanoparticles of a certain size range, and/or certain characteristic(s), for example, nanoparticles associated with ovarian cancer. In some such embodiments, prior to a capture assay, a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample) may be pre-processed to remove contaminants, including, *e.g.*, but not limited to soluble proteins and interfering entities such as, *e.g.*, cell debris. For example, in some embodiments, nanoparticles are purified from a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample) of a subject, for example, using size exclusion chromatography. In some such embodiments, nanoparticles can be directly purified from a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample), for example, using size exclusion chromatography, which in some embodiments may remove at least 90% or higher (including, *e.g.*, at least 93%, 95%, 97%, 99% or higher) of soluble proteins and other interfering agents such as, *e.g.*, cell debris.

[0405] In some embodiments, a capture assay comprises a step of contacting a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample) with at least one capture agent comprising a target-capture moiety that binds to at least one or more provided biomarkers of a target biomarker signature for ovarian cancer. In some embodiments, a capture assay may be multiplexed, which comprises a step of contacting a biological sample (*e.g.*, a bodily fluid-derived sample such as a blood-derived sample) with a set of capture agents, each capture agent comprising a target-capture moiety that binds to a distinct provided biomarker of a target biomarker signature for ovarian cancer. In some embodiments, a target-capture moiety is directed to an extracellular vesicle-associated surface biomarker or surface biomarker (*e.g.*, ones as described and/or utilized herein).

[0406] In some embodiments, such a target-capture moiety may be immobilized on a solid substrate. Accordingly, in some embodiments, a capture agent employed in a capture assay is or comprises a solid substrate comprising at least one or more (*e.g.*, 1, 2, 3, 4, 5, or more) target-capture moiety conjugated thereto, each target-capture moiety directed to an extracellular vesicle-associated surface biomarker or surface biomarker (*e.g.*, ones as described and/or utilized herein). A solid substrate may be provided in a form that is suitable for capturing nanoparticles having a size range of interest that includes extracellular vesicles and does not interfere with downstream handling, processing, and/or detection. For example, in some embodiments, a solid substrate may be or comprise a bead (*e.g.*, a magnetic bead). In some embodiments, a solid substrate may be or comprise a surface. For example, in some embodiments, such a surface may be a capture surface of an assay chamber (including, *e.g.*, a tube, a well, a microwell, a plate, a filter, a membrane, a matrix, *etc.*). In some embodiments, a capture agent is or comprises a magnetic bead comprising a target-capture moiety conjugated thereto.

[0407] In some embodiments, a detection assay is performed to detect one or more provided biomarkers of a target biomarker signature for ovarian cancer (*e.g.*, ones that are different from ones targeted in a capture assay) in nanoparticles that are captured by a capture assay (*e.g.*, as described above). In some embodiments, a detection assay may comprise immuno-PCR. In some embodiments, an immuno-PCR may involve at least one probe targeting a single provided biomarker (*e.g.*, ones described herein) of a target biomarker signature for ovarian cancer. In some embodiments, an immuno-PCR may involve a plurality of (*e.g.*, at least two, at least three, at least four, or more) probes directed to different epitopes of the same biomarker (*e.g.*, ones described herein) of a target biomarker signature. In some embodiments, an immuno-PCR may involve a plurality of (*e.g.*, at least two, at least three, at least four, or more) probes, each directed to a different provided biomarker described herein.

[0408] In some embodiments, a detection assay may comprise reverse transcription polymerase chain reaction (RT-PCR). In some embodiments, an RT-PCR may involve at least one primer/probe set targeting a single provided biomarker described herein. In some embodiments, an RT-PCR may involve a plurality of (*e.g.*, at least two, at least three, at least four, or more) primer/probe sets, each set directed to a different provided biomarker described herein.

[0409] In some embodiments, a detection assay may comprise a proximity-ligation-immuno quantitative polymerase chain reaction (pliq-PCR), for example, to determine co-localization of one or more provided biomarkers of a target biomarker signature for ovarian cancer within nanoparticles having a size range of interest that includes extracellular vesicles (*e.g.*, captured extracellular vesicles that express at least one extracellular vesicle-associated surface biomarker).

[0410] In some embodiments, a detection assay employs a target entity detection system that was developed by Applicant and described in U.S. Application No. 16/805,637 (issued as

US11,085,089), and International Application PCT/US2020/020529 (published as WO2020180741), both filed February 28, 2020 and entitled “Systems, Compositions, and Methods for Target Entity Detection” (the “’089 patent” and the “’529 application”; both of which are incorporated herein by reference in their entirety) which are, in part, based on interaction and/or co-localization of a target biomarker signature in individual nanoparticles. For example, such a target entity detection system (as described in the ’089 patent and ’529 application and also further described below in the section entitled “*Provided Target Entity Detection Systems and Methods Involving the Same*”) can detect in a sample (e.g., in a biological, environmental, or other sample), in some embodiments at a single entity level, entities of interest (e.g., biological or chemical entities of interest, such as nanoparticles or analytes) comprising at least one or more (e.g., at least two or more) targets (e.g., molecular targets). Those skilled in the art, reading the present disclosure, will recognize that provided target entity detection systems are useful for a wide variety of applications and/or purposes, including, e.g., for detection of ovarian cancer. For example, in some embodiments, provided target entity detection systems may be useful for medical applications and/or purposes. In some embodiments, provided target entity detection systems may be useful to screen (e.g., regularly screen) individuals (e.g., in some embodiments which may be asymptomatic individuals, or in some embodiments which may be individuals experiencing one or more symptoms associated with ovarian cancer, or in some embodiments which may be individuals at risk for ovarian cancer such as, e.g., individuals with a hereditary risk for ovarian cancer and/or life-history-associated risk factor, or post-menopausal individuals) for a disease or condition (e.g., ovarian cancer). In some embodiments, provided target entity detection systems may be useful to screen (e.g., regularly screen) individuals (e.g., in some embodiments which may be asymptomatic individuals, or in some embodiments which may be individuals experiencing one or more symptoms associated with ovarian cancer, or in some embodiments which may be individuals at risk for ovarian cancer such as, e.g., individuals with a hereditary risk for ovarian cancer and/or life-history-associated risk factor, or post-menopausal individuals) for different types of cancer (e.g., for a plurality of different cancers, one of which may be ovarian cancer). In some embodiments, provided target entity detection systems 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 embodiments, provided target entity detection systems may be useful as a companion diagnostic in conjunction with a disease treatment (e.g., treatment of ovarian cancer).

[0411] In some embodiments, a plurality of (e.g., at least two or more) detection assays may be performed to detect a plurality of biomarkers (e.g., at least two or more) of one or more target biomarker signatures for ovarian cancer (e.g., ones that are different from ones targeted in a capture assay) in nanoparticles, e.g., ones that are captured by a capture assay (e.g., as described above). For example, in some embodiments, a plurality of detection assays may comprise (i) a provided target

entity detection system or a system described in the '089 patent and '529 application and/or described herein; and (ii) immuno-PCR. In some embodiments, a plurality of detection assays may comprise (i) a provided target entity detection system or a system described in the '089 patent and '529 application; and (ii) RT-PCR.

[0412] For example, in some embodiments, a subject's sample comprising extracellular vesicles may be first subjected to detection of surface biomarkers (e.g., as described herein) using a target entity detection system or a system described in the '089 patent and '529 application and/or described herein and then subjected to a lysis buffer to release intravesicular analytes, followed by a nucleic acid assay (e.g., in some embodiments RT-qPCR) for detection of one or more intravesicular RNA biomarkers. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise an mRNA transcript encoded by a biomarker gene described herein. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise a microRNA. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise an orphan noncoding RNA. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise a long noncoding RNA. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise a piwi-interacting RNA. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise a circular RNA. In some embodiments, one or more intravesicular RNA biomarkers may be or comprise a small nucleolar RNA.

V. Provided Target Entity Detection Systems and Methods Involving the Same

[0413] In some embodiments, a target entity detection system that can be useful in a detection assay for one or more provided biomarkers of one or more target biomarker signatures for ovarian cancer includes a plurality of detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, or more detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise 2-50 detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise 2-30 detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise 2-25 detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise 5-30 detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, such a system may comprise 5-25 detection probes each for a specific target (e.g., a provided biomarker of a target biomarker signature). In some embodiments, at least two of such detection probes in a set may be directed to the same biomarker of

a target biomarker signature. In some embodiments, at least two of such detection probes in a set may be directed to the same epitope of the same biomarker of a target biomarker signature. In some embodiments, at least two of such detection probes in a set may be directed to different epitopes of the same biomarker of a target biomarker signature.

[0414] In some embodiments, detection probes appropriate for use in a target entity detection system provided herein may be used for detection of a single disease or condition, *e.g.*, ovarian cancer. In some embodiments, detection probes appropriate for use in a target entity detection system provided herein may permit detection of at least two or more diseases or conditions, *e.g.*, one of which is ovarian cancer. In some embodiments, detection probes appropriate for use in a target entity detection system provided herein may permit detection of ovarian cancer of certain subtypes including, *e.g.*, but not limited to, high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, and/or mucinous ovarian cancer. In some embodiments, detection probes appropriate for use in a target entity detection system provided herein may permit detection of ovarian cancer of certain stages, including, *e.g.*, stage I, stage II, stage III, and/or stage IV. Accordingly, in some embodiments, detection probes appropriate for use in a target entity detection system provided herein may comprise a plurality (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, or more) of sets of detection probes, wherein each set is directed to detection of a different disease or a different type of disease or condition. For example, in some embodiments, detection probes appropriate for use in a target entity detection system provided herein may comprise a plurality (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, or more) of sets of detection probes, wherein in some embodiments, each set is directed to detection of a different type of cancer, one of which is ovarian cancer, or in some embodiments, each set is directed to detection of ovarian cancer of various subtypes and/or stages.

Detection probes

[0415] In some embodiments, a detection probe as provided and/or utilized herein comprises a target-binding moiety and an oligonucleotide domain coupled to the target-binding moiety. In some embodiments, an oligonucleotide domain coupled to a target-binding moiety may comprise a double-stranded portion and a single-stranded overhang extended from at least one end of the oligonucleotide domain. In some embodiments, an oligonucleotide domain coupled to a target-binding moiety may comprise a double-stranded portion and a single-stranded overhang extended from each end of the oligonucleotide domain. In some embodiments, detection probes may be suitable for proximity-ligation-immuno quantitative polymerase chain reaction (pliq-PCR) and be referred to as pliq-PCR detection probes.

A. Target-binding moieties

[0416] A target-binding moiety that is coupled to an oligonucleotide domain is an entity or an agent that specifically binds to a target (*e.g.*, a provided biomarker of a target biomarker signature; those skilled in the art will appreciate that, where the target biomarker is a particular form or moiety/component, the target-binding moiety specifically binds to that form or moiety/component). In some embodiments, a target-binding moiety may have a binding affinity (*e.g.*, as measured by a dissociation constant) for a target (*e.g.*, molecular target) of at least about 10^{-4} M, at least about 10^{-5} M, at least about 10^{-6} M, at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, or lower. Those skilled in the art will appreciate that, in some cases, binding affinity (*e.g.*, as measured by a dissociation constant) may be influenced by non-covalent intermolecular interactions such as hydrogen bonding, electrostatic interactions, hydrophobic and Van der Waals forces between the two molecules. Alternatively or additionally, binding affinity between a ligand and its target molecule may be affected by the presence of other molecules. Those skilled in the art will be familiar with a variety of technologies for measuring binding affinity and/or dissociation constants in accordance with the present disclosure, including, *e.g.*, but not limited to ELISAs, surface plasmon resonance (SPR) assays, Luminex Single Antigen (LSA) assays, bio-layer interferometry (BLI) (*e.g.*, Octet) assays, grating-coupled interferometry, and spectroscopic assays.

[0417] In some embodiments, a target-binding moiety is assessed for off-target interactions. In some embodiments, a target-binding moiety is assessed using immunocapture followed by mass spectrometry (*e.g.*, to reveal off target binding events in a complex sample). In some embodiments, a target-binding moiety is assessed using protein or glycan arrays, *e.g.*, where many thousands of human proteins or glycans are arrayed on a chip and an antibody's binding is profiled across all available targets (*e.g.*, a specific antibody will only bind to a target of interest). In some embodiments, a target-binding moiety is assessed using traditional immunoassays such as Western blot. In some embodiments, a target-binding moiety is assessed for generic off-target non-specific binding (*e.g.*, binding to other antibodies, DNA, lipids, *etc.*). In some embodiments, such generic off-target non-specific binding may be measured and identified using a negative control to identify a false positive signal (*e.g.*, suggesting that one or more antibodies bind non-specifically, and not to a target).

[0418] In some embodiments, a target-binding moiety may be or comprise an agent of any chemical class such as, for example, a carbohydrate, a nucleic acid, a lipid, a metal, a polypeptide, a small molecule, *etc.*, and/or a combination thereof. In some embodiments, a target-binding moiety may be or comprise an affinity agent such as an antibody, affimer, aptamer, lectin, siglec, *etc.* In some embodiments, a target-binding moiety is or comprises an antibody agent, *e.g.*, an antibody agent that specifically binds to a target or an epitope thereof, *e.g.*, a provided biomarker of a target biomarker signature for ovarian cancer or an epitope thereof. In some embodiments, a target-binding

moiety for a provided biomarker may be a commercially available. In some embodiments, a target-binding moiety for a provided biomarker may be designed and created for the purpose of use in assays as described herein. In some embodiments, a target-binding moiety is or comprises an aptamer, *e.g.*, an aptamer that specifically binds to a target or an epitope thereof, *e.g.*, a provided biomarker of a target biomarker signature for ovarian cancer or an epitope thereof. In some embodiments, a target-binding moiety is or comprises a lectin or siglec that specifically binds to a carbohydrate-dependent marker as provided herein. In some embodiments, a target-binding moiety is or comprises an affimer molecule that specifically binds to a target or an epitope thereof, *e.g.*, a provided biomarker of a target biomarker signature for ovarian cancer or an epitope thereof. In some embodiments, such an affimer molecule can be or comprise a peptide or polypeptide that binds to a target or an epitope thereof (*e.g.*, as described herein) with similar specificity and affinity to that of a corresponding antibody. In some embodiments, a target may be or comprise a target that is associated with ovarian cancer. For example, in some such embodiments, 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 embodiments, a cancer-associated target can be or comprise a target that is typically associated with cancers. In some embodiments, a cancer-associated target can be or comprise a target that is associated with cancers of a specific tissue, *e.g.*, ovarian cancer. In some embodiments, a cancer-associated target can be or comprise a target that is specific to a particular cancer, *e.g.*, a particular ovarian cancer.

[0419] In some embodiments, a target-binding moiety recognizes and specifically binds to a target present in a biological entity (including, *e.g.*, but not limited to cells and/or nanoparticles). For example, in some embodiments, a target-binding moiety may recognize and specifically bind to a tumor-associated antigen or epitope thereof. In some embodiments, a tumor-associated antigen may be or comprise an antigen that is associated with a cancer such as, for example, skin cancer, brain cancer (including, *e.g.*, glioblastoma), breast cancer, colorectal cancer, liver cancer, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, and skin cancer. In some embodiments, a target-binding moiety may recognize a tumor antigen associated with ovarian cancer (*e.g.*, high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer). In some embodiments, a target-binding moiety may recognize a tumor antigen associated with high-grade serous ovarian cancer (HGSOC).

[0420] In some embodiments, a target-binding moiety may specifically bind to an intravesicular target, *e.g.*, a provided intravesicular protein or RNA (*e.g.*, mRNA, snRNA, miRNA, siRNA, orphan noncoding RNA, long noncoding RNA, or piwi-interacting RNA). In some embodiments, a target-binding moiety may specifically bind to a surface target that is present on/within nanoparticles, *e.g.*, a surface biomarker present on ovarian cancer-associated nanoparticles.

[0421] In some embodiments, a target-binding moiety is directed to a biomarker for a specific condition or disease (*e.g.*, cancer), which biomarker is or has been determined, for example, by analyzing a population or library (*e.g.*, tens, hundreds, thousands, tens of thousands, hundreds of thousands, or more) of patient biopsies and/or patient data to identify such a biomarker (*e.g.*, a predictive biomarker).

[0422] In some embodiments, a relevant biomarker may be one identified and/or characterized, for example, via data analysis. In some embodiments, for example, a diverse set of data (*e.g.*, in some embodiments comprising one or more of bulk RNA sequencing, single-cell RNA (scRNA) sequencing, mass spectrometry, histology, post-translational modification data, *in vitro* and/or *in vivo* experimental data) can be analyzed through machine learning and/or computational modeling to identify biomarkers (*e.g.*, predictive markers) that are highly specific to a disease or condition (*e.g.*, cancer).

[0423] In some embodiments, a target-binding moiety is directed to a tissue-specific target, for example, a target that is associated with a specific tissue such as, for example, brain, breast, colon, ovary and/or other tissues associated with a female reproductive system, pancreas, prostate and/or other tissues associated with a male reproductive system, liver, lung, and skin. In some embodiments, such a tissue-specific target may be associated with a normal healthy tissue and/or a diseased tissue, such as a tumor. In some embodiments, a target-binding moiety is directed to a target that is specifically associated with a normal healthy condition of a subject.

[0424] In some embodiments, individual target binding entities utilized in a plurality of detection probes (*e.g.*, as described and/or utilized herein) are directed to different targets. In some embodiments, such different targets may represent different marker proteins or polypeptides. In some embodiments, such different targets may represent different epitopes of the same marker proteins or polypeptides. In some embodiments, two or more individual target binding entities utilized in a plurality of detection probes (*e.g.*, as described and/or utilized herein) may be directed to the same target.

[0425] In some embodiments, individual target binding entities utilized in a plurality of detection probes for detection of ovarian cancer may be directed to different target biomarkers of a target biomarker signature for ovarian cancer (*e.g.*, ones as described in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*” above). By way of example, in some embodiments, at least two detection probes in a plurality may have their target binding entities directed to MUC16 and FOLR1, respectively. In some embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and MUC16, respectively. In some embodiments, at least two detection probes in a plurality may have their target binding entities directed to BCAM and BST2, respectively. In some embodiments, at least two detection probes in a plurality may have their target binding entities

directed to BST2 and FOLR1, respectively. In some embodiments, at least two detection probes in a plurality may have their target binding entities directed to BST2 and MUC1, respectively.

[0426] In some embodiments, individual target binding entities utilized in a plurality of detection probes for detection of ovarian cancer may be directed to the same target biomarker of a target biomarker signature for ovarian cancer (*e.g.*, ones as described in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*” above). In some embodiments, such target binding entities may be directed to the same or different epitopes of the same target biomarker of such a target biomarker signature for ovarian cancer. By way of example only, in some embodiments, at least two detection probes in a plurality may have their target binding entities each directed to MUC16 (*e.g.*, in its intact trans-membrane protein form, and/or at the same epitope or at different epitopes). In some embodiments, at least two detection probes in a plurality may have their target binding entities each directed to a FOLR1 peptide (*e.g.*, at the same epitope or at different epitopes).

B. Oligonucleotide domains

[0427] In some embodiments, an oligonucleotide domain for use in accordance with the present disclosure (*e.g.*, that may be coupled to a target-binding moiety) may comprise a double-stranded portion and a single-stranded overhang extended from one or both ends of the oligonucleotide domain. In some embodiments where an oligonucleotide domain comprises a single-stranded overhang extended from each end, a single-stranded overhang is extended from a different strand of a double-stranded portion. In some embodiments where an oligonucleotide domain comprises a single-stranded overhang extended from one end of the oligonucleotide domain, the other end of the oligonucleotide domain may be a blunt end.

[0428] In some embodiments, an oligonucleotide domain may comprise ribonucleotides, deoxyribonucleotides, synthetic nucleotide residues that are capable of participating in Watson-Crick type or analogous base pair interactions, and any combinations thereof. In some embodiments, an oligonucleotide domain is or comprises DNA. In some embodiments, an oligonucleotide domain is or comprises peptide nucleic acid (PNA).

[0429] In some embodiments, an oligonucleotide may have a length that is determined, at least in part, for example, by, *e.g.*, the physical characteristics of an entity of interest (*e.g.*, biological entity such as extracellular vesicles) to be detected, and/or selection and localization of molecular targets in an entity of interest (*e.g.*, biological entity such as extracellular vesicles) to be detected. In some embodiments, 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 an entity of interest (*e.g.*, biological entity such as extracellular vesicles), the first single-stranded overhang and the second single-stranded overhang are in sufficiently close proximity to permit interaction (*e.g.*, hybridization).

between the single-stranded overhangs. For example, when an entity of interest (*e.g.*, biological entity) is an extracellular vesicle (*e.g.*, an exosome), oligonucleotide domains of detection probes can each independently have a length such that their respective single-stranded overhangs are in sufficiently close proximity to anneal or interact with each other when the corresponding detection probes are bound to the same extracellular vesicle. For example, in some embodiments, oligonucleotide domains of detection probes for use in detecting extracellular vesicles (*e.g.*, an exosome) may each independently have a length of about 20 nm to about 200 nm, about 40 nm to about 500 nm, about 40 nm to about 300 nm, or about 50 nm to about 150 nm. In some embodiments, oligonucleotide domains of detection probes for use in detecting extracellular vesicles (*e.g.*, an exosome) may each independently have a length of about 20 nm to about 200 nm. In some embodiments, lengths of oligonucleotide domains of detection probes in a set can each independently vary to increase and/or maximize the probability of them finding each other when they simultaneously bind to the same entity of interest. Such oligonucleotide domains designed for use in detection probes for detecting extracellular vesicles can also be used in detection probes for detecting nanoparticles having a size range of interest that includes extracellular vesicles.

[0430] Accordingly, in some embodiments, an oligonucleotide domain for use in technologies provided herein may have a length in the range of about 20 up to about 1000 nucleotides. In some embodiments, an oligonucleotide domain may have a length in the range of about 30 up to about 1000 nucleotides, In some embodiments, an oligonucleotide domain may have a length in the range of about 30 to about 500 nucleotides, from about 30 to about 250 nucleotides, from about 30 to about 200 nucleotides, from about 30 to about 150 nucleotides, from about 40 to about 150 nucleotides, from about 40 to about 125 nucleotides, from about 40 to about 100 nucleotides, from about 40 to about 60 nucleotides, from about 50 to about 90 nucleotides, from about 50 to about 80 nucleotides. In some embodiments, an oligonucleotide domain may have a length of at least 20 or more nucleotides, including, *e.g.*, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 250, at least 500, at least 750, at least 1000 nucleotides or more. In some embodiments, an oligonucleotide domain may have a length of no more than 1000 nucleotides or lower, including, *e.g.*, no more than 900, no more than 800, no more than 700, no more than 600, no more than 500, no more than 400, no more than 300, no more than 200, no more than 100, no more than 90, no more than 80, no more than 70, no more than 60, no more than 50, no more than 40 nucleotides, no more than 30 nucleotides, no more than 20 nucleotides or lower.

[0431] In some embodiments, an oligonucleotide domain may have a length of about 20 nm to about 500 nm. In some embodiments, an oligonucleotide domain may have a length of about 20 nm to about 400 nm, about 30 nm to about 200 nm, about 50 nm to about 100 nm, about 30 nm to about 70 nm, or about 40 nm to about 60 nm. In some embodiments, an oligonucleotide domain may

have a length of at least about 20 nm or more, including, *e.g.*, at least about 30 nm, at least about 40 nm, at least about 50 nm, at least about 60 nm, at least about 70 nm, at least about 80 nm, at least about 90 nm, at least about 100 nm, at least about 200 nm, at least about 300 nm, at least about 400 nm or more. In some embodiments, an oligonucleotide domain may have a length of no more than 1000 nm or lower, including, *e.g.*, no more than 900 nm, no more than 800 nm, no more than 700 nm, no more than 600 nm, no more than 500 nm, no more than 400 nm, no more than 300 nm, no more than 200 nm, no more than 100 nm or lower.

[0432] In some embodiments, a double-stranded portion of an oligonucleotide domain for use in technologies provided herein may have a length in the range of about 30 up to about 1000 nucleotides. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length in the range of about 30 to about 500 nucleotides, from about 30 to about 250 nucleotides, from about 30 to about 200 nucleotides, from about 30 to about 150 nucleotides, from about 40 to about 150 nucleotides, from about 40 to about 125 nucleotides, from about 40 to about 100 nucleotides, from about 50 to about 90 nucleotides, from about 50 to about 80 nucleotides. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of at least 30 or more nucleotides, including, *e.g.*, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 250, at least 500, at least 750, at least 1000 nucleotides or more. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of no more than 1000 nucleotides or lower, including, *e.g.*, no more than 900, no more than 800, no more than 700, no more than 600, no more than 500, no more than 400, no more than 300, no more than 200, no more than 100, no more than 90, no more than 80, no more than 70, no more than 60, no more than 50, no more than 40 nucleotides or lower.

[0433] In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of about 20 nm to about 500 nm. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of about 20 nm to about 400 nm, about 30 nm to about 200 nm, about 50 nm to about 100 nm, about 30 nm to about 70 nm, or about 40 nm to about 60 nm. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of at least about 20 nm or more, including, *e.g.*, at least about 30 nm, at least about 40 nm, at least about 50 nm, at least about 60 nm, at least about 70 nm, at least about 80 nm, at least about 90 nm, at least about 100 nm, at least about 200 nm, at least about 300 nm, at least about 400 nm or more. In some embodiments, a double-stranded portion of an oligonucleotide domain may have a length of no more than 1000 nm or lower, including, *e.g.*, no more than 900 nm, no more than 800 nm, no more than 700 nm, no more than 600 nm, no more than 500 nm, no more than 400 nm, no more than 300 nm, no more than 200 nm, no more than 100 nm or lower.

[0434] In some embodiments, a double-stranded portion of an oligonucleotide domain is characterized in that when detection probes are connected to each other through hybridization of

respective complementary single-stranded overhangs (*e.g.*, as described and/or utilized herein), the combined length of the respective oligonucleotide domains (including, if any, a linker that links a target-binding moiety to an oligonucleotide domain) is long enough to allow respective target binding entities to substantially span the full characteristic length (*e.g.*, diameter) of an entity of interest (*e.g.*, an extracellular vesicle). For example, in some embodiments where nanoparticles (*e.g.*, extracellular vesicles) are entities of interest, a combined length of oligonucleotide domains (including, if any, a linker that links a target-binding moiety to an oligonucleotide domain) of detection probes may be approximately 50 to 200 nm, when the detection probes are fully connected to each other.

[0435] In some embodiments, a double-stranded portion of an oligonucleotide domain may comprise a binding site for a primer. In some embodiments, such a binding site for a primer may comprise a nucleotide sequence that is designed to reduce or minimize the likelihood for miss-priming or primer dimers. Such a feature, in some embodiments, can decrease the lower limit of detection and thus increase the sensitivity of systems provided herein. In some embodiments, a binding site for a primer may comprise a nucleotide sequence that is designed to have a similar annealing temperature as another primer binding site.

[0436] In some embodiments, a double-stranded portion of an oligonucleotide domain may comprise a nucleotide sequence designed to reduce or minimize overlap with nucleic acid sequences (*e.g.*, DNA and/or RNA sequences) typically associated with genome and/or gene transcripts (*e.g.*, genomic DNA and/or RNA, such as mRNA of genes) of a subject (*e.g.*, a human subject). Such a feature, in some embodiments, may reduce or minimize interference of any genomic DNA and/or mRNA transcripts of a subject that may be present (*e.g.*, as contaminants) in a sample during detection.

[0437] In some embodiments, a double-stranded portion of an oligonucleotide domain may have a nucleotide sequence designed to reduce or minimize formation of self-dimers, homo-dimers, or hetero-dimers.

[0438] In some embodiments, a single-stranded overhang of an oligonucleotide domain for use in technologies provided herein may have a length of about 2 to about 20 nucleotides. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of about 2 to about 15 nucleotides, from about 2 to about 10 nucleotides, from about 3 to about 20 nucleotides, from about 3 to about 15 nucleotides, from about 3 to about 10 nucleotides. In some embodiments, a single-stranded overhang can have at least 1 to 5 nucleotides in length. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of at least 2 or more nucleotides, including, *e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20 nucleotides, or more. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of

no more than 20 nucleotides or lower, including, *e.g.*, no more than 15, no more than 14, no more than 13, no more than 12, no more than 11, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4 nucleotides or lower.

[0439] In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of about 1 nm to about 10 nm. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of about 1 nm to about 5 nm. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of at least about 0.5 nm or more, including, *e.g.*, at least about 1 nm, at least about 1.5 nm, at least about 2 nm, at least about 3 nm, at least about 4 nm, at least about 5 nm, at least about 6 nm, at least about 7 nm, at least about 8 nm, at least about 9 nm, at least about 10 nm or more. In some embodiments, a single-stranded overhang of an oligonucleotide domain may have a length of no more than 10 nm or lower, including, *e.g.*, no more than 9 nm, no more than 8 nm, no more than 7 nm, no more than 6 nm, no more than 5 nm, no more than 4 nm, no more than 3 nm, no more than 2 nm, no more than 1 nm or lower.

[0440] A single-stranded overhang of an oligonucleotide domain is designed to comprise a nucleotide sequence that is complementary to at least a portion of a single-stranded overhang of a second detection probe such that a double-stranded complex comprising a first detection probe and a second detection probe can be formed through hybridization of the complementary single-stranded overhangs. In some embodiments, nucleotide sequences of complementary single-stranded overhangs are selected for optimal ligation efficiency in the presence of an appropriate nucleic acid ligase. In some embodiments, a single-stranded overhang has a nucleotide sequence preferentially selected for efficient 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, *e.g.*, as described in Song *et al.*, “Enzyme-guided DNA sewing architecture” *Scientific Reports* 5: 17722 (2015), which is incorporated herein by reference for the purpose described herein.

[0441] When two detection probes couple together through hybridization of respective complementary single-stranded overhangs, their respective oligonucleotide domains comprising the hybridized single-stranded overhangs can, in some embodiments, have a combined length of about 90%-110% or about 95%-105% of a characteristic length (*e.g.*, diameter) of an entity of interest (*e.g.*, a biological entity). For example, in some embodiments when a biological entity is an exosome, the combined length can be about 50 nm to about 200 nm, or about 75 nm to about 150 nm, or about 80 nm to about 120 nm.

C. Coupling between a target-binding moiety and an oligonucleotide domain

[0442] An oligonucleotide domain and a target-binding moiety can be coupled together in a detection probe by a covalent linkage, and/or by a non-covalent association (such as, *e.g.*, a protein-

protein interaction such as streptavidin-biotin interaction and/or an ionic interaction). In some embodiments, a detection probe appropriate for use in accordance with the present disclosure is a conjugate molecule comprising a target-binding moiety and an oligonucleotide domain, where the two components are typically covalently coupled to each other, *e.g.*, directly through a bond, or indirectly through one or more linkers. In some embodiments, a target-binding moiety is coupled to one of two strands of an oligonucleotide domain by a covalent linkage (*e.g.*, directly through a bond or indirectly through one or more linkers) and/or by a non-covalent association (such as, *e.g.*, a protein-protein interaction such as streptavidin-biotin interaction and/or ionic interaction).

[0443] Where linkers are employed, in some embodiments, linkers are chosen to provide for covalent attachment of a target-binding moiety to one or both strands of an oligonucleotide domain through selected linkers. In some embodiments, linkers are chosen such that the resulting covalent attachment of a target-binding moiety to one or both strands of an oligonucleotide domain maintains the desired binding affinity of the target-binding moiety for its target. In some embodiments, linkers are chosen to enhance binding specificity of a target-binding moiety for its target. Linkers and/or conjugation methods of interest may vary widely depending on a target-binding moiety, *e.g.*, its size and/or charges. In some embodiments, linkers are biologically inert.

[0444] A variety of linkers and/or methods for coupling a target-binding moiety to an oligonucleotide is known to one of ordinary skill in the art and can be used in accordance with the present disclosure. In some embodiments, a linker can comprise a spacer group at either end with a reactive functional group at either end capable of covalent attachment to a target-binding moiety. Examples of spacer groups that can be used in linkers include, but are not limited to, aliphatic and unsaturated hydrocarbon chains (including, *e.g.*, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, or longer), spacers containing heteroatoms such as oxygen (*e.g.*, ethers such as polyethylene glycol) or nitrogen (polyamines), peptides, carbohydrates, cyclic or acyclic systems that may contain heteroatoms. Non-limiting examples of a reactive functional group to facilitate covalent attachment include nucleophilic functional groups (*e.g.*, amines, alcohols, thiols, and/or hydrazides), electrophilic functional groups (*e.g.*, aldehydes, esters, vinyl ketones, epoxides, isocyanates, and/or maleimides), functional groups capable of cycloaddition reactions, forming disulfide bonds, or binding to metals. In some embodiments, exemplary reactive functional groups, but are not limited to, primary and secondary amines, hydroxamic acids, N-hydroxysuccinimidyl (NHS) esters, dibenzocyclooctyne (DBCO)-NHS esters, azido-NHS esters, azidoacetic acid NHS ester, propargyl-NHS ester, trans-cyclooctene-NHS esters, N-hydroxysuccinimidyl carbonates, oxycarbonylimidazoles, nitrophenylesters, trifluoroethyl esters, glycidyl ethers, vinylsulfones, maleimides, azidobenzoyl hydrazide, N-[4-(p-azidosalicylamino)butyl]-3'-[2'-pyridylidithio]propionamid, bis-sulfosuccinimidyl suberate, dimethyladipimide, disuccinimidyltartrate, N-maleimidobutyryloxysuccinimide ester, N-hydroxy

sulfosuccinimidyl-4-azidobenzoate, N-succinimidyl [4-azidophenyl]-1,3'-dithiopropionate, N-succinimidyl [4-iodoacetyl]aminobenzoate, glutaraldehyde, and succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate, 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC), and any combinations thereof.

[0445] In some embodiments, a target-binding moiety (*e.g.*, a target binding antibody agent) is coupled or conjugated to one or both strands of an oligonucleotide domain using N-hydroxysuccinimide (NHS) ester chemistry. NHS esters react with free primary amines and result in stable covalent attachment. In some embodiments, a primary amino group can be positioned at a terminal end with a spacer group, *e.g.*, but not limited to an aliphatic and unsaturated hydrocarbon chain (*e.g.*, a C6 or C12 spacer group).

[0446] In some embodiments, a target-binding moiety (*e.g.*, a target-binding affinity agent) can be coupled or conjugated to one or both strands of an oligonucleotide domain using a site-specific conjugation method known in the art, *e.g.*, to enhance the binding specificity of conjugated target-binding moiety (*e.g.*, conjugated target-binding affinity agent). Examples of a site-specific conjugation method include, but are not limited to coupling or conjugation through a disulfide bond, C-terminus, carbohydrate residue or glycan, and/or unnatural amino acid labeling. In some embodiments where a target-binding moiety is or comprises an affinity agent, an oligonucleotide can be coupled or conjugated to the target-binding moiety via at least one or more free amine groups present in the target-binding moiety. In some embodiments, an oligonucleotide can be coupled or conjugated to a target-binding moiety that is or comprises an affinity agent via at least one or more reactive thiol groups present in the target-binding moiety. In some embodiments, an oligonucleotide can be coupled or conjugated to a target-binding moiety that is or comprises an antibody agent or a peptide aptamer via at least one or more carbohydrate residues present in the target-binding moiety.

[0447] In some embodiments, a plurality of oligonucleotides (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least ten, or more) can be coupled or conjugated to a target-binding moiety (*e.g.*, a target binding antibody agent).

Exemplary duplex target entity detection system

[0448] In some embodiments, a target entity detection system as provided by the present disclosure (and useful, for example, for detecting, *e.g.*, at a single entity level, nanoparticles associated with ovarian cancer) may comprise a first population of first detection probes (*e.g.*, as described and/or utilized herein) for a provided target biomarker (*e.g.*, ones described herein) and a second population of second detection probes (*e.g.*, as described and/or utilized herein) for a provided target biomarker (*e.g.*, ones described herein). In some embodiments, the first detection probes and the second detection probes are directed to the same provided target biomarker. In some

embodiments, the first detection probes and the second detection probes are directed to different provided target biomarkers.

[0449] *Fig. 2* illustrates an exemplary duplex target entity detection system for detecting, at a single entity level, an entity of interest (*e.g.*, biological entity such as an extracellular vesicle) comprising (i) at least one target (*e.g.*, a provided biomarker of a target biomarker signature for ovarian cancer) which expression level is high enough such that two molecules of the same target (*e.g.*, a provided biomarker of a target biomarker signature for ovarian cancer) are found in close proximity, or (ii) at least two or more distinct targets (*e.g.*, provided biomarkers of a target biomarker signature for ovarian cancer). A first detection probe comprises a first target-binding moiety (*e.g.*, directed to a target cancer marker 1) 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. As shown in *Fig. 2*, a first oligonucleotide domain may be resulted from hybridization of a longer strand (strand 3) and a shorter strand (strand 1), thereby forming a double-stranded portion and a single-stranded overhang at one end. In some embodiments, a first target-binding moiety (*e.g.*, directed to target cancer marker 1) is coupled (*e.g.*, covalently coupled) to a 5' end or 3' end of a strand of a first oligonucleotide domain (*e.g.*, strand 1). In some embodiments, a 5' end or 3' end of a strand that is coupled to a first target-binding moiety may be modified with a linker (*e.g.*, as described and/or utilized herein with or without a spacer group). In some embodiments, a 5' end of another strand of a first oligonucleotide domain (*e.g.*, strand 3) has a free phosphate group.

[0450] In the embodiment depicted in *Fig. 2*, a second detection probe comprises a second target-binding moiety (*e.g.*, directed to a target cancer marker 2) 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. As shown in *Fig. 2*, a second oligonucleotide domain may be resulted from hybridization of a longer strand (strand 4) and a shorter strand (strand 2), thereby forming a double-stranded portion and a single-stranded overhang at one end. In some embodiments, a second target-binding moiety (*e.g.*, directed to a target cancer marker 2) is coupled (*e.g.*, covalently coupled) to a 5' end of a strand of a second oligonucleotide domain (*e.g.*, strand 2). In some embodiments, a 5' end of a strand that is coupled to a second target-binding moiety may be modified with a linker (*e.g.*, as described and/or utilized herein with or without a spacer group). In some embodiments, a 5' end of another strand of a second oligonucleotide domain (*e.g.*, strand 4) has a free phosphate group.

[0451] At least portions of a first single-stranded overhang and a second single-stranded overhang are complementary to each other such that they can hybridize to form a double-stranded complex when they are in sufficiently close proximity, *e.g.*, when a first detection probe and a

second detection probe simultaneously bind to the same entity of interest (e.g., biological entity such as extracellular vesicle). In some embodiments, a first single-stranded overhang and a second single-stranded overhang have equal lengths such that when they hybridize to form a double-stranded complex, there is no gap (other than a nick to be ligated) between their respective oligonucleotide domains and each respective target-binding moiety is located at an opposing end of the double-stranded complex. For example, in some embodiments, a double-stranded complex forms before ligation occurs, wherein the double-stranded complex comprises a first detection probe and a second detection probe coupled to each other through direct hybridization of their respective single-stranded overhangs (e.g., having 4 nucleotides in length), wherein each respective target-binding moiety (e.g., directed to a target cancer marker 1 and a target cancer marker 2, respectively) is present at opposing ends of the double-stranded complex. In such embodiments, both strands of the double-stranded complex (comprising a nick between respective oligonucleotide domains) are ligatable, e.g., for amplification and detection. In some embodiments, a double-stranded complex (e.g., before ligation occurs) can comprise an entity of interest (e.g., a biological entity such as an extracellular vesicle), wherein a first target-binding moiety (e.g., directed to a target cancer marker 1) and a second target-binding moiety (e.g., directed to a target cancer marker 2) are simultaneously bound to the entity of interest.

[0452] In some embodiments of a duplex target entity detection system for detection of ovarian cancer (e.g., HGSOE), a first target-binding moiety of a first detection probe may be directed to a first target surface biomarker (e.g., ones provided in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*”), while a second target-binding moiety of a second detection probe may be directed to a second target surface biomarker (e.g., ones provided in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*”). In some embodiments, a first target-binding moiety of a first detection probe may be directed to a first target intravesicular biomarker (e.g., ones provided in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*”), while a second target-binding moiety of a second detection probe may be directed to a second target intravesicular biomarker (e.g., ones provided in the section entitled “*Provided Biomarkers and/or Target Biomarker Signatures for Detection of Ovarian Cancer*”). In some embodiments, the first target-binding moiety and the second target-binding moiety may be directed to the same or different epitopes of the same target surface biomarker or of the same target intravesicular biomarker. In some embodiments, the first target-binding moiety and the second target-binding moiety may be directed to the different target surface biomarkers or different target intravesicular biomarkers.

[0453] In some embodiments of a duplex target entity detection system for detection of ovarian cancer, a first detection probe comprises a first target-binding moiety directed to a surface

biomarker (*e.g.*, as described herein) conjugated to a first oligonucleotide domain; whereas a second detection probe comprises a second target-binding moiety detected to the same surface biomarker conjugated to a second oligonucleotide domain. In some such embodiments, the first target-binding moiety and the second target-binding moiety can be directed to the same or different epitope(s) of a surface biomarker (*e.g.*, as described herein). In some embodiments, the double stranded portion of a first oligonucleotide domain and a second oligonucleotide domain may be the same. In some embodiments, the double stranded portion of a first oligonucleotide domain and a second oligonucleotide domain may be different.

[0454] In some embodiments of a duplex target entity detection system for detection of ovarian cancer, a first detection probe comprises a first target-binding moiety directed to a surface biomarker (*e.g.*, as described herein) conjugated to a first oligonucleotide domain; whereas a second detection probe comprises a second target-binding moiety directed to a different surface biomarker (*e.g.*, as described herein) conjugated to a second oligonucleotide domain. In some embodiments, the double-stranded portion of a first oligonucleotide domain and a second oligonucleotide domain may be the same. In some embodiments, the double-stranded portion of a first oligonucleotide domain and a second oligonucleotide domain may be different.

[0455] In some embodiments, a duplex target entity detection system for detection of ovarian cancer may comprise at least two distinct sets of detection probes. For example, in some embodiments, each set may be directed to a distinct target biomarker signature comprising one or more target biomarkers (*e.g.*, ones described herein). In some embodiments, each set may be directed to a distinct combination of target biomarkers for ovarian cancer. For example, in some embodiments, a duplex target entity detection system may comprise at least two sets of detection probes, wherein a first set comprises at least two detection probes each directed to a distinct biomarker, and a second set comprises at least two detection probes each directed to a distinct biomarker.

[0456] In some embodiments, a duplex target entity detection system for detection of ovarian cancer (*e.g.*, HGSOc) may comprise at least three distinct sets of detection probes. For example, in some embodiments, each set may be directed to a distinct target biomarker signature comprising one or more target biomarkers (*e.g.*, ones described herein). In some such embodiments, at least one set may be directed to a single target biomarker (*e.g.*, ones described herein). In some such embodiments, at least one set may be directed to a combination of at least two distinct target biomarkers (*e.g.*, combinations of at least two target biomarkers described herein).

[0457] In some embodiments, a duplex target entity detection system comprising at least two distinct sets of detection probes may also comprise a capture assay comprising a capture agent directed to an extracellular vesicle-associated surface biomarker.

[0458] In some embodiments, any combination of biomarker probes (*e.g.*, a biomarker signature) including capture probes or detection probes as described herein may be utilized in combination with any other set of biomarker probes (*e.g.*, a biomarker signature) including capture probes or detection probes as described herein.

Exemplary triplex or multiplex ($n \geq 3$) target entity detection system

[0459] In some embodiments, a target entity detection system as provided by the present disclosure (and useful, for example, for detecting, *e.g.*, at a single entity level, nanoparticles associated with ovarian cancer) may comprise n populations of distinct detection probes (*e.g.*, as described and/or utilized herein), wherein $n \geq 3$. For example, in some embodiments when $n = 3$, a target entity detection system may comprise a first detection probe (*e.g.*, as described and/or utilized herein) for a first target, a population of a second detection probe (*e.g.*, as described and/or utilized herein) for a second target, and a population of a third detection probe (*e.g.*, as described and/or utilized herein) for a third target.

[0460] *Fig. 3* illustrates an exemplary triplex target entity detection system for detecting, at a single entity level, an entity of interest (*e.g.*, a biological entity such as an extracellular vesicle) comprising three distinct molecular targets. A first detection probe comprises a first target-binding moiety (*e.g.*, anti-cancer marker 1 antibody agent) 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. As shown in *Fig. 3*, a first oligonucleotide domain may be resulted from hybridization of a longer strand (strand 8) and a shorter strand (strand 1), thereby forming a double-stranded portion and a single-stranded overhang at one end. In some embodiments, a first target-binding moiety (*e.g.*, anti-cancer marker 1 antibody agent) is coupled (*e.g.*, covalently coupled) to a 5' end of a strand of a first oligonucleotide domain (*e.g.*, strand 1). In some embodiments, a 5' end of a strand that is coupled to a first target-binding moiety may be modified with a linker (*e.g.*, as described and/or utilized herein with or without a spacer group). In some embodiments, a 5' end of another strand of a first oligonucleotide domain (*e.g.*, strand 8) has a free phosphate group.

[0461] In the embodiment depicted in *Fig. 3*, a second detection probe comprises a second target-binding moiety (*e.g.*, anti-cancer marker 3 antibody agent) 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. As shown in *Fig. 3*, a second oligonucleotide domain may be resulted from hybridization of a longer strand (strand 4) and a shorter strand (strand 2), thereby forming a double-stranded portion and a single-stranded overhang at one end. In some embodiments, a second target-binding moiety (*e.g.*, anti-cancer marker 3 antibody agent) is coupled (*e.g.*,

covalently coupled) to a 5' end of a strand of a second oligonucleotide domain (*e.g.*, strand 2). In some embodiments, a 5' end of a strand that is coupled to a second target-binding moiety may be modified with a linker (*e.g.*, as described and/or utilized herein with or without a spacer group). In some embodiments, a 5' end of another strand of a second oligonucleotide domain (*e.g.*, strand 4) has no free phosphate group.

[0462] A third detection probe comprises a third target-binding moiety (*e.g.*, anti-cancer marker 2 antibody agent) and a third oligonucleotide domain coupled to the third target-binding moiety, the third oligonucleotide domain comprising a third double-stranded portion and a single-stranded overhang extended from each end of the third oligonucleotide domain. For example, a single-stranded overhang is extended from one end of a strand of a third oligonucleotide domain while another single-stranded overhang is extended from an opposing end of a different strand of the third oligonucleotide domain. As shown in *Fig. 3*, a third oligonucleotide domain may be resulted from hybridization of portions of two strands (*e.g.*, strands 9 and 10), thereby forming a double-stranded portion and a single-stranded overhang at each end. For example, a single-stranded overhang (3A) is formed at a 5' end of strand 9 of a third detection probe, wherein the 5' end of strand 9 has a free phosphate group. Additionally, a single-stranded overhang (3B) is formed at a 5' end of strand 10 of the same third detection probe and a third target-binding moiety (*e.g.*, anti-target 2 antibody agent) is also coupled (*e.g.*, covalently coupled) to the 5' end of strand 10. In some embodiments, a 5' end of a strand (*e.g.*, strand 10) that is coupled to a third target-binding moiety may be modified with a linker (*e.g.*, as described and/or utilized herein with or without a spacer group).

[0463] When all three detection probes are in sufficiently close proximity, *e.g.*, when all three detection probes simultaneously bind to the same entity of interest (*e.g.*, biological entity), (i) at least a portion of a single-stranded overhang (*e.g.*, 3A) of a third detection probe is hybridized to a corresponding complementary portion of a single-stranded overhang of a second detection probe, and (ii) at least a portion of another single-stranded overhang (*e.g.*, 3B) of the third detection probe is hybridized to a corresponding complementary portion of a single-stranded overhang of a first detection probe. As a result, a double-stranded complex comprising all three detection probes coupled to each other in a linear arrangement is formed by direct hybridization of corresponding single-stranded overhangs. See, *e.g.*, *Fig. 3*.

[0464] In some embodiments involving use of at least three or more ($n \geq 3$) detection probes in provided technologies, 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 such embodiments, 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 to form a ligated template. *e.g.*, for amplification and detection. See, *e.g.*, **Fig. 3** (using three detection probes), **Fig. 4** (using four detection probes), and **Fig. 5** (using *n* detection probes).

[0465] In some embodiments 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. The size of the gap is large enough that the strand becomes non-ligatable in the presence of a nucleic acid ligase. In some embodiments, the gap may be 2-8 nucleotides in size or 2-6 nucleotides in size. In some embodiments, the gap is 6 nucleotides in size. In some embodiments, the overlap (hybridization region between single-stranded overhangs) can be 2-15 nucleotides in length or 4-10 nucleotides in length. In some embodiments, the overlap (hybridization region between single-stranded overhangs) is 8 nucleotides in length. The size of the gap and/or hybridization region are selected to provide an optimum signal separation from a ligated template (comprising no internal target binding moieties) and non-ligated template (comprising at least one internal target-binding moiety). It should be noted that while **Figs. 3-5** do not show binding of detection probes to an entity of interest (*e.g.*, a biological entity), a double-stranded complex (*e.g.*, before ligation occurs) can comprise an entity of interest (*e.g.*, a biological entity such as extracellular vesicles), wherein at least three or more target binding moieties are simultaneously bound to the entity of interest.

[0466] In some embodiments, selection of a combination (*e.g.*, a set) of detection probes (*e.g.*, number of detection probes and/or specific biomarkers) for use in a target entity detection system provided herein (*e.g.*, a duplex, triplex or multiplex target entity detection system described herein) is based on, for example, a desired specificity and/or a desired sensitivity that is deemed to be optimal for a particular application. For example, in some embodiments, a combination of detection probes is selected for detection of ovarian cancer (*e.g.*, for stage I, II, III, or IV) such that it provides a specificity of at least 95% or higher, including, *e.g.*, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, at least 99.7%, at least 99.8% or higher. In some embodiments, a combination of detection probes is selected for detection of ovarian cancer (*e.g.*, for stage I, II, III, or IV) such that it provides a sensitivity of at least 30% or higher, including, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or higher. In some embodiments, a combination of detection probes is selected for detection of ovarian cancer (*e.g.*, for stage I, II, III, or IV) such that it provides a positive predictive value of at least 8% or higher, including, *e.g.*, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or higher. In some embodiments, a combination of detection probes is selected for detection of ovarian cancer (*e.g.*, for stage I, II, III, or IV) such that it provides a positive predictive value of at least 2% or higher, including, *e.g.*, at least 3%, at least 4%, at least 5%, at least

6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or higher. In some embodiments, a combination of detection probes is selected for detection of ovarian cancer (*e.g.*, for stage I, II, III, or IV) such that it provides a limit of detection (LOD) below 1×10^7 EV/mL sample or lower, including, *e.g.*, below 7×10^6 EV/mL sample, below 6×10^6 EV/mL sample, below 5×10^6 EV/mL sample, below 4×10^6 EV/mL sample, below 3×10^6 EV/mL sample, below 2×10^6 EV/mL sample, below 1×10^6 EV/mL sample, or lower. In some embodiments, such ovarian cancer detection assay may be used to detect different subtypes of ovarian cancer including, *e.g.*, but not limited to high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer. In some embodiments, such ovarian cancer detection assay may be used to detect ovarian cancer of an epithelial origin. In some embodiments, such ovarian cancer detection assay may be used to detect high-grade serous ovarian cancer (HGSOC).

[0467] In some embodiments, a combination (*e.g.*, a set) of detection probes, rather than individual detection probes, confers specificity to detection of a disease, disorder, or condition (*e.g.*, a particular ovarian cancer and/or a stage of ovarian cancer as described herein), for example, one or more individual probes may be directed to a target that itself is not specific to ovarian cancer. For example, in some embodiments, a useful combination of detection probes in a target entity detection system provided herein (*e.g.*, a duplex, triplex or multiplex target entity detection system described herein) may comprise at least one detection probe directed to a target specific for the relevant disease, disorder, or condition (*i.e.*, a target that is specific to the relevant disease, disorder, or condition), and may further comprise at least one detection probe directed to a target that is not necessarily or completely specific for the relevant disease, disorder, or condition (*e.g.*, that may also be found on some or all cells that are healthy, are not of the particular disease, disorder, or condition, and/or are not of the particular disease 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 detection of the relevant disease, disorder, or condition (*i.e.*, sufficiently distinguish biological entities for detection that are associated with the relevant disease, disorder, or condition from other biological entities not of interest for detection), the set is useful in accordance with certain embodiments of the present disclosure.

[0468] In some embodiments, a target entity detection system provided herein (*e.g.*, a duplex, triplex or multiplex target entity detection system described herein) can comprise at least one or more (*e.g.*, at least 2 or more) control probes (in addition to target-specific detection probes, *e.g.*, as described and/or utilized herein, for example, in some embodiments to recognize disease-specific biomarkers such as cancer-specific biomarkers and/or tissue-specific biomarkers). In some

embodiments, a control probe is designed such that its binding to an entity of interest (*e.g.*, a biological entity) inhibits (completely or partially) generation of a detection signal.

[0469] In some embodiments, a control probe comprises a control binding moiety and an oligonucleotide domain (*e.g.*, as described and/or utilized herein) coupled to the control binding moiety, the oligonucleotide domain comprising a double-stranded portion and a single-stranded overhang extended from one end of the oligonucleotide domain. A control binding moiety is an entity or moiety that binds to a control reference. In some embodiments, a control reference can be or comprise a biomarker that is preferentially associated with a normal healthy cell. In some embodiments, a control reference can be or comprise a biomarker preferentially associated from a non-target tissue. In some embodiments, inclusion of a control probe can selectively remove or minimize detectable signals generated from false positives (*e.g.*, entities of interest comprising a control reference, optionally in combination with one or more targets to be detected). In some embodiments, a control reference can be or comprises CD63, CD81, or combinations thereof. In some embodiments, a control probe can be used to normalize detection signals from samples (*e.g.*, ovarian cancer samples and/or reference samples) so as to improve separation of positive signal (from ovarian cancer samples) from a baseline signal (*e.g.*, from reference samples). Other control probes described in U.S. Application No. 16/805,637 (published as US2020/0299780; issued as US11,085,089), and International Application PCT/US2020/020529 (published as WO2020180741), both filed February 28, 2020 and entitled “Systems, Compositions, and Methods for Target Entity Detection,” the entire contents of each application are incorporated herein by reference in their entirety, can be useful in provided target entity detections systems.

[0470] In some embodiments, a target entity detection system provided herein (*e.g.*, a duplex, triplex or multiplex target entity detection system described herein) can comprise at least one or more (*e.g.*, at least 2 or more) cross-reactivity markers (in addition to target-specific detection probes, *e.g.*, as described and/or utilized herein, for example, in some embodiments to recognize disease-specific biomarkers such as cancer-specific biomarkers and/or tissue-specific biomarkers).

[0471] In some embodiments, a cross-reactivity marker may comprise a biomarker that has a high expression level in normal ovarian tissue, but low in cancerous ovarian tissue.

[0472] In some embodiments, the present disclosure provides insights, among other things, that detection probes as described or utilized herein may non-specifically bind to a solid substrate surface and some of them may remain in an assay sample even after multiple washes to remove any excess or unbound detection probes; and that such non-specifically bound detection probes may come off from the solid substrate surface and become free-floating in a ligation reaction, thus allowing them to interact with one another to generate a non-specific ligated template that produces an undesirable background signal. Accordingly, in some embodiments, a target entity detection system provided herein (*e.g.*, a duplex, triplex, or multiplex target entity detection described herein)

can comprise at least one or more (*e.g.*, at least 2 or more) inhibitor oligonucleotides that are designed to capture residual detection probes that are not bound to an entity of interest but remain as free agents in a ligation reaction, thereby preventing such free-floating detection probes from interacting with other free-floating complementary detection probes to produce an undesirable background signal. In some embodiments, an inhibitor oligonucleotide may be or comprise a single-stranded or double-stranded oligonucleotide comprising a binding domain for a single-stranded overhang of a detection probe (*e.g.*, as described or utilized herein), wherein the inhibitor oligonucleotide does not comprise a primer binding site. The absence of such a primer binding site in an inhibitor oligonucleotide prevents a primer from binding to a non-specific ligated template resulting from ligation of a detectable probe to an inhibitor oligonucleotide, thereby reducing or inhibiting the non-specific ligated template from amplification and/or detection, *e.g.*, by polymerase chain reaction.

[0473] In some embodiments, an inhibitor oligonucleotide comprises a binding domain for a single-stranded overhang of a detection probe (*e.g.*, as described or utilized herein), wherein the binding domain is or comprises a nucleotide sequence that is substantially complementary to the single-stranded overhang of the detection probe such that a free, unbound detection probe having a complementary single-stranded overhang can bind to the binding domain of the inhibitor oligonucleotide. In some embodiments, an inhibitor oligonucleotide may have a hairpin at one end. In some embodiments, an inhibitor oligonucleotide may be a single-stranded oligonucleotide comprising at one end a binding domain for a single-stranded overhang of a detection probe, wherein a portion of the single-stranded oligonucleotide can self-hybridize to form a hairpin at another end.

[0474] In some embodiments, a target entity detection system provided herein (*e.g.*, a duplex, triplex or multiplex target entity detection system described herein) does not comprise a connector oligonucleotide that associates an oligonucleotide domain of a detection probe with an oligonucleotide domain of another detection probe. In some embodiments, a connector oligonucleotide is designed to bridge oligonucleotide domains of any two detection probes that would not otherwise interact with each other when they bind to an entity of interest. In some embodiments, a connector oligonucleotide is designed to hybridize with at least a portion of an oligonucleotide domain of a detection probe and at least a portion of an oligonucleotide domain of another detection probe. A connector oligonucleotide can be single-stranded, double-stranded, or a combination thereof. A connector oligonucleotide is free of any target-binding moiety (*e.g.*, as described and/or utilized herein) or control binding moiety. In at least some embodiments, no connector oligonucleotides are necessary to indirectly connect oligonucleotide domains of detection probes; in some embodiments, such connector oligonucleotides are not utilized, in part because detection probes as provided and/or utilized herein are designed such that their respective oligonucleotide domains have a sufficient length to reach and interact with each other when they are

in sufficiently close proximity, *e.g.*, when the detection probes simultaneously bind to an entity of interest (*e.g.*, a biological entity such as an extracellular vesicle).

Methods of using provided target entity detection systems

[0475] Provided target entity detection systems are useful in detecting an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) in a sample (*e.g.*, in a biological, environmental, or other sample) for various applications and/or purposes associated with detection of ovarian cancer. Accordingly, some aspects provided herein relate to methods of using a plurality of (*e.g.*, at least 2, at least 3, or more) detection probes appropriate for use in accordance with the present disclosure. In some embodiments, a method comprises contacting an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) in a sample (*e.g.*, a biological sample such as a blood-derived sample from a human subject) with a set of detection probes comprising at least 2 or more (including, *e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or more) detection probes as described and/or utilized herein. In some embodiments, a method comprises subjecting a sample comprising an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) to a target entity detection system (*e.g.*, as provided herein). A plurality of detection probes (*e.g.*, at least two or more) can be added to a sample comprising an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) at the same time or at different times (*e.g.*, sequentially). In some embodiments, a method may comprise, prior to contacting with a plurality of detection probes, contacting a sample comprising an entity of interest with at least one capture agent directed to an extracellular vesicle-associated surface biomarker.

[0476] In certain embodiments, a provided target entity detection system for use in a method described herein may comprise a plurality of (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or more) distinct sets (*e.g.*, combinations) of detection probes (*e.g.*, as described herein). In some embodiments, a method comprises contacting an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) in a sample (*e.g.*, a biological sample such as a blood-derived sample from a human female subject) with a plurality of sets of detection probes, wherein each set may comprise at least 2 or more (including, *e.g.*, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or more) detection probes as described and/or utilized herein. In some embodiments, a method comprises subjecting a sample comprising an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) to a target entity detection system (*e.g.*, as provided herein). A plurality of detection probes and/or detection probe combinations (*e.g.*, at least two or more) can be added to a

sample comprising an entity of interest (*e.g.*, a biological entity such as extracellular vesicles) at the same time or at different times (*e.g.*, sequentially). In some embodiments, a method may comprise, prior to contacting with a plurality of detection probes, contacting a sample comprising an entity of interest with at least one capture agent directed to an extracellular vesicle-associated surface biomarker.

[0477] In some embodiments, the relationship between results (*e.g.*, Ct values and/or relative number of ligated nucleic acid templates (*e.g.*, ligated DNA templates)) from profiling one or more biomarker combinations in a sample can be combined with clinical information (including, *e.g.*, but not limited to patient age, past medical history, plasma CA-125, *etc.*) and/or other information to better classify patients with or at risk for ovarian cancer. Various classification algorithms can be used to interpret the relationship between multiple variables to increase an assay's sensitivity and/or specificity. In some embodiments, such algorithms include, but are not limited to, logistic regression models, support vector machines, gradient boosting machines, random forest algorithms, Naive Bayes algorithms, K-nearest neighborhood algorithms, and combinations thereof. In some embodiments, performance (*e.g.*, accuracy) of assays described herein can be improved, *e.g.*, by selection of biomarker combinations (*e.g.*, as described herein), selection of other factors or variables (*e.g.*, clinical information and/or lifestyle information) to include an algorithm, and/or selection of the type of algorithm itself.

[0478] In certain embodiments, technologies described herein utilize a predictive algorithm that is trained and validated using data sets as described herein. In certain embodiments, technologies described herein are utilized to generate a risk score using an algorithm created from training samples which is designed to take into account results from at least two, *e.g.*, at least two, at least 3, at least 4, at least 5, or more than 5 separate assays comprising biomarker signatures (*e.g.*, as described herein). In certain embodiments, an algorithm-generated risk score can be generated at least in part using diagnostic data (*e.g.*, raw and/or normalized Ct values) from at least one individual assay (*e.g.*, individual biomarker signature). In certain embodiments, a reference threshold can be included within a risk score. In certain embodiments, multiple threshold levels denoting multiple different degrees of ovarian cancer risk may be included in a risk score. In some embodiments, separate target biomarker signature assays may be performed as individual assays in a series of assays, and individual assays may be weighted equally or differently in a predictive algorithm. In some embodiments, for example, weighting of individual assays combined in an algorithm (*e.g.*, a cohort of biomarker assays) may be determined by a number of factors including but not limited to the sensitivity of an individual assay, the specificity of an individual assay, the reproducibility of an individual assay, the variability of an individual assay, the positive predictive value of an individual assay, and/or the lowest limit of detection of a specific assay. In some embodiments, a cohort of biomarker assays may be ranked according to a characteristic (*e.g.*, sensitivity, specificity, lowest

limit of detection *etc.*) and the biomarker assays may then be weighted based upon their relative rank.

[0479] In some embodiments, a risk score generated by an algorithm (as described herein) can be presented in a suitable manner, *e.g.*, on a nominal scale, *e.g.*, on a scale of 0-100 reflecting a number of likelihoods, *e.g.*, including but not limited to the likelihood a subject has ovarian cancer, the likelihood a subject will develop ovarian cancer, and/or the likely stage of ovarian cancer. In some embodiments, a higher risk score can demonstrate that there is an increasing likelihood of disease pathology, *e.g.*, lower to higher values may reflect healthy controls, benign controls, stage I, stage II, stage III, and stage IV ovarian cancers. In some embodiments, a risk score can be utilized to reduce the potential of cross reactivity of technologies as described herein when compared with other cancer types.

[0480] In some embodiments, a risk score may be generated from a combination of data derived from assays as described herein coupled with other applicable diagnostic data such as age, life history, TVUS results, CA-125 levels, blood biomarker test results, or any combination thereof. In some embodiments, a risk score provides predictive value above and beyond that of conventional standard of care diagnostic assay predictive values, *e.g.*, higher than predictive values provided by TVUS and/or CA-125 assays utilized in isolation or in combination with another diagnostic assay. In some embodiments, a risk score may be generated that has high specificity for ovarian cancers (*e.g.*, HGSOC) and has low sensitivity for other cancers.

[0481] In some embodiments, a risk score may have an associated clinical cutoff for detection of ovarian cancer. For example, in some embodiments, a risk score's clinical cutoff for detection may require an assay that yields at least 40%, *e.g.*, at least 50%, at least 60%, or greater sensitivity for detection of both early and late stage ovarian cancer and has a minimum of 90% specificity, *e.g.*, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater specificity in a generally healthy population of female subjects (*e.g.*, aged 40 to 85 years of age) or in a population of subjects with hereditary risk. In some embodiments, sensitivity and specificity targets are the approximate lower bounds of the two-sided 95% confidence interval for the targeted 77% sensitivity and 99.5% specificity.

[0482] In some embodiments, a training study is performed to provide the necessary data required to program a risk score algorithm. In some embodiments, such a training study may comprise a cohort of samples from a range of suppliers, including at least commercial suppliers, biorepositories (*e.g.*, biobanks), purpose driven studies, and/or physicians. In some embodiments, a training study may comprise positive samples from high-grade serous ovarian cancer patients (*e.g.*, stage I, stage II, stage III, and/or stage IV), positive control samples from high-grade serous ovarian cancer cell lines, negative samples from benign gynecological tumor patients (*e.g.*, ovarian tumor, uterine tumor, *etc.*) including patients with benign adnexal masses, negative samples from non-

ovarian cancer patients (also referred to herein as off-target cancers, including, *e.g.*, brain cancer, breast cancer, colorectal cancer, endometrial cancer, lung adenocarcinoma, melanoma, non-Hodgkin's lymphoma, pancreatic cancer, skin cancer, *etc.*), negative samples from inflammatory condition patients (*e.g.*, Crohn's disease, endometriosis, diabetes type II, lupus, pancreatitis, rheumatoid arthritis, ulcerative colitis, *etc.*), negative samples from healthy patients, or any combination thereof. In some embodiments, a training study comprises expression of a pre-determined set comprising a plurality of biomarker combinations as described herein (*e.g.*, in some embodiments biomarker combinations as shown in **Table 8**) for various types of samples described herein (including, *e.g.*, samples from early-stage ovarian cancer and/or late-stage ovarian cancer, samples from normal healthy subjects, samples from patients with benign adnexal masses, samples from off-target cancers and/or inflammatory conditions, or combinations thereof). In some embodiments, a training study may comprise samples from patients of any appropriate age range, *e.g.*, <31 years old, 31-40 years old, 41-50 years old, 51-60 years old, 61-70 years old, 71-80 years old, or >80 years old. In some embodiments, a training study may comprise samples from patients of any race/ethnicity/descent, (*e.g.*, Caucasians, Africans, Asians *etc.*).

[0483] In some embodiments, a training set that involves data from various types of samples can be used to create a risk score algorithm and a validation study can be performed to assess or improve the performance of the trained risk score algorithm. In some embodiments, such a training set and/or validation study may comprise a cohort of samples from a range of suppliers, including at least commercial suppliers, biorepositories (*e.g.*, biobanks), purpose driven studies, and/or physicians. In some embodiments, a training set and/or validation study may comprise positive samples from high-grade serous ovarian cancer patients (*e.g.*, stage I, stage II, stage III, and/or stage IV), positive control samples from high-grade serous ovarian cancer cell lines, negative samples from benign gynecological tumor patients (*e.g.*, ovarian tumor, uterine tumor, *etc.*), negative samples from non-ovarian cancer patients (*e.g.*, brain cancer, breast cancer, colorectal cancer, endometrial cancer, lung adenocarcinoma, melanoma, non-Hodgkin's lymphoma, pancreatic cancer, skin cancer, *etc.*), negative samples from inflammatory condition patients (*e.g.*, Crohn's disease, endometriosis, diabetes type II, lupus, pancreatitis, rheumatoid arthritis, ulcerative colitis, *etc.*), negative samples from healthy patients, or any combination thereof. In some embodiments, a training set and/or validation study may comprise samples from patients of any appropriate age range, *e.g.*, <31 years old, 31-40 years old, 41-50 years old, 51-60 years old, 61-70 years old, 71-80 years old, or >80 years old. In some embodiments, a training set and/or validation study may comprise samples from patients of any race/ethnicity/descent, (*e.g.*, Caucasians, Africans, Asians, *etc.*).

[0484] In certain embodiments, at least one target biomarker signature comprising at least one surface biomarker (*e.g.*, extracellular vesicle-associated surface biomarker) and at least one

(including, *e.g.*, at least two, or more) target biomarker (which may be or comprise any of surface biomarkers described herein) may be embodied in an ovarian cancer detection assay. In some such embodiments, at least one capture agent is directed to the extracellular vesicle-associated surface biomarker, and at least one set of detection probes is directed to one or more of such target biomarkers described herein. In some embodiments, a target biomarker signature may further comprise an internal biomarker (*e.g.*, intravesicular biomarker and/or intravesicular RNA biomarkers) and/or a surface biomarker described in WO 2021/146659, the entire contents of which are incorporated by reference for purposes described herein.

[0485] In certain embodiments, at least two (including, *e.g.*, at least three or more) distinct target biomarker signatures each comprising at least one extracellular vesicle-associated surface biomarker and at least one (including, *e.g.*, at least two, or more) target biomarker (which may be or comprise any of surface biomarkers described herein) may be embodied in an ovarian cancer detection assay. In some embodiments, a target biomarker signature may further comprise an internal biomarker (*e.g.*, intravesicular biomarker and/or intravesicular RNA biomarkers) and/or a surface biomarker described in WO 2021/146659, the entire contents of which are incorporated by reference for purposes described herein. By way of example only in some embodiments, an ovarian cancer detection assay can utilize at least two target biomarker signatures (*e.g.*, as described herein) for ovarian cancer, wherein a first target biomarker signature comprises at least Biomarker A and Biomarker B; and a second target biomarker signature comprises at least Biomarker A and Biomarker C, wherein combinations of Biomarker A, Biomarker B, and/or Biomarker C correspond to certain biomarker combinations described herein (*e.g.*, as shown in *Table 1*). In some embodiments, a capture agent directed to Biomarker A, which in some embodiments, may be immobilized on solid substrates, *e.g.*, beads such as magnetic beads), is used to capture nanoparticles from a patient's biological sample (*e.g.*, a blood-derived sample). A first aliquot of captured nanoparticles is subjected to a first set of detection probes directed to at least Biomarker B, while a second aliquot of captured nanoparticles is subjected to a second set of detection probes directed to at least Biomarker C.

[0486] In some embodiments, an ovarian cancer detection assay can utilize at least three target biomarker signatures (*e.g.*, as described herein) for ovarian cancer, wherein a first target biomarker signature comprises at least Biomarker A and Biomarker B; a second target biomarker signature comprises at least Biomarker A and Biomarker C; and a third target biomarker signature comprises Biomarker B and Biomarker C, wherein combinations of Biomarker A, Biomarker B, and/or Biomarker C correspond to certain biomarker combinations described herein (*e.g.*, as shown in *Table 1*). In some embodiments, an aliquot of a patient's biological sample (*e.g.*, a blood-derived sample) is subjected to a first capture agent directed to Biomarker A, which in some embodiments, may be immobilized on solid substrates, *e.g.*, beads such as magnetic beads) for capture of

nanoparticles for further analysis. A first aliquot of Biomarker A-captured nanoparticles is subjected to a first set of detection probes each directed to Biomarker B, while a second aliquot of Biomarker A-captured nanoparticles is subjected to a second set of detection probes each directed to Biomarker C. In some embodiments, another aliquot of such a patient's biological sample is subjected to a second capture agent directed to Biomarker B, which in some embodiments, may be immobilized on solid substrates, *e.g.*, beads such as magnetic beads) for capture of nanoparticles for further analysis. Biomarker B-captured nanoparticles are then subjected to a set comprising a first detection probe directed to Biomarker B and a second detection probe directed to Biomarker C.

[0487] In some embodiments, each distinct target biomarker signature may have a different pre-determined cutoff value for individually determining whether a sample is positive for ovarian cancer. In some embodiments, a sample is determined to be positive for ovarian cancer if assay readout is above at least one of cutoff values for a plurality of (*e.g.*, at least 2 or more) target biomarker signatures. In some embodiments, a diagnostic value or a risk score cutoff can be determined based on a plurality of (*e.g.*, at least 2, at least 3 or more) target biomarker signatures.

[0488] Accordingly, in some embodiments, a sample can be divided into aliquots such that a different capture agent and/or a different set of detection probes (*e.g.*, each directed to detection of a distinct disease or condition) can be added to a different aliquot. In such embodiments, provided technologies can be implemented with one aliquot at a time or multiple aliquots at a time (*e.g.*, for parallel assays to increase throughput).

[0489] In some embodiments, amount of detection probes that is added to a sample provides a sufficiently low concentration of detection probes in a mixture to ensure that the detection probes will not randomly come into close proximity with one another in the absence of binding to an entity of interest (*e.g.*, biological entity), at least not to any great or substantial degree. As such, in many embodiments, when detection probes simultaneously bind to the same entity of interest (*e.g.*, biological entity) through the binding interaction between respective targeting binding moieties of the detection probes and the binding sites of an entity of interest (*e.g.*, a biological entity), the detection probes come into sufficiently close proximity to one another to form double-stranded complex (*e.g.*, as described herein). In some embodiments, the concentration of detection probes in a mixture following combination with a sample may range from about 1 fM to 1 μ M, such as from about 1 pM to about 1 nM, including from about 1 pM to about 100 nM.

[0490] In some embodiments, the concentration of an entity of interest (*e.g.*, a biological entity) in a sample is sufficiently low such that a detection probe binding to one entity of interest (*e.g.*, a biological entity) will not randomly come into close proximity with another detection probe binding to another entity of interest (*e.g.*, biological entity) in the absence of respective detection probes binding to the same entity of interest (*e.g.*, biological entity), at least not to any great or substantial degree. By way of example only, the concentration of an entity of interest (*e.g.*, biological

entity) in a sample is sufficiently low such that a first target detection probe binding to a non-target entity of interest (*e.g.*, a non-cancerous biological entity such as an extracellular vesicle comprising a first target) will not randomly come into close proximity with another different target detection probe that is bound to another non-target entity of interest (*e.g.*, a non-cancerous biological entity such as an extracellular vesicle), at least not to any great or substantial degree, to generate a false positive detectable signal.

[0491] Following contacting an entity of interest (*e.g.*, biological entity) in a sample with a set of detection probes, such a mixture may be incubated for a period of time sufficient for the detection probes to bind corresponding targets (*e.g.*, molecular targets), if present, in the entity of interest to form a double-stranded complex (*e.g.*, as described herein). In some embodiments, such a mixture is incubated for a period of time ranging from about 5 min to about 5 hours, including from about 30 min to about 2 hours, at a temperature ranging from about 10 to about 50 °C, including from about 20 °C to about 37 °C.

[0492] A double-stranded complex (resulted from contacting an entity of interest such as a biological entity with detection probes) can then be subsequently contacted with a nucleic acid ligase to perform nucleic acid ligation of a free 3' end hydroxyl and 5' end phosphate end of oligonucleotide strands of detection probes, thereby generating a ligated template comprising oligonucleotide strands of at least two or more detection probes. In some embodiments, prior to contacting an assay sample comprising a double-stranded complex with a nucleic acid ligase, at least one or more inhibitor oligonucleotide (*e.g.*, as described herein) can be added to the assay sample such that the inhibitor oligonucleotide can capture any residual free-floating detection probes that may otherwise interact with each other during a ligation reaction.

[0493] As is known in the art, ligases catalyze the formation of a phosphodiester bond between juxtaposed 3'-hydroxyl and 5'-phosphate termini of two immediately adjacent nucleic acids when they are annealed or hybridized to a third nucleic acid sequence to which they are complementary. Any known nucleic acid ligase (*e.g.*, DNA ligases) may be employed, including but not limited to temperature sensitive and/or thermostable ligases. Non-limiting examples of temperature sensitive ligases include bacteriophage T4 DNA ligase, bacteriophage T7 ligase, and *E. coli* ligase. Non-limiting examples of thermostable ligases include Taq ligase, Tth ligase, and Pfu ligase. Thermostable ligase may be obtained from thermophilic or hyperthermophilic organisms, including but not limited to, prokaryotic, eukaryotic, or archaeal organisms. In some embodiments, a nucleic acid ligase is a DNA ligase. In some embodiments, a nucleic acid ligase can be a RNA ligase.

[0494] In some embodiments, in a ligation step, a suitable nucleic acid ligase (*e.g.*, a DNA ligase) and any reagents that are necessary and/or desirable are combined with the reaction mixture and maintained under conditions sufficient for ligation of the hybridized ligation oligonucleotides to

occur. Ligation reaction conditions are well known to those of skill in the art. During ligation, a reaction mixture, in some embodiments, may be maintained at a temperature ranging from about 20° C to about 45° C, such as from about 25° C to about 37° C for a period of time ranging from about 5 minutes to about 16 hours, such as from about 1 hour to about 4 hours. In yet other embodiments, a reaction mixture may be maintained at a temperature ranging from about 35° C to about 45° C, such as from about 37° C to about 42° C, *e.g.*, at or about 38 ° C, 39° C, 40° C or 41° C, for a period of time ranging from about 5 minutes to about 16 hours, such as from about 1 hour to about 10 hours, including from about 2 to about 8 hours.

[0495] Detection of such a ligated template can provide 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 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. In some embodiments, a detectable level is a level that is above a reference level, *e.g.*, by at least 10% or more, including, *e.g.*, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or more. In some embodiments, 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. Conversely, 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). Those of skill in the art will appreciate that a threshold that separates a detectable level from a non-detectable level may be determined based on, for example, a desired sensitivity level, and/or a desired specificity level that is deemed to be optimal for each application and/or purpose. For example, in some embodiments, a specificity of 99.7% may be achieved using a system provided herein, for example by setting a threshold that is three standard deviations above a reference level (*e.g.*, a level observed in a negative control sample, such as, *e.g.*, a sample derived from one or more normal healthy individuals). Additionally or alternatively, those of skill in the art will appreciate that a threshold of a detectable level (*e.g.*, as reflected by a detection signal intensity) may be 1 to 100-fold above a reference level.

[0496] In some embodiments, a method provided herein comprises, following ligation, detecting a ligated template, *e.g.*, as a measure of the presence and/or amount of an entity of interest in a sample. In various embodiments, detection of a ligated template may be qualitative or quantitative. As such, in some embodiments where detection is qualitative, a method provides a reading or evaluation, *e.g.*, assessment, of whether or not an entity of interest (*e.g.*, a biological entity) comprising at least two or more targets (*e.g.*, molecular targets) is present in a sample being assayed. In other embodiments, a method provides a quantitative detection of whether an entity of interest (*e.g.*, a biological entity) comprising at least two or more targets (*e.g.*, molecular targets) is

present in a sample being assayed, *e.g.*, an evaluation or assessment of the actual amount of an entity of interest (*e.g.*, a biological entity) comprising at least two or more targets (*e.g.*, molecular targets) in a sample being assayed. In some embodiments, such quantitative detection may be absolute or relative.

[0497] A ligated template formed by using technologies provided herein may be detected by an appropriate method known in the art. Those of skill in the art will appreciate that appropriate detection methods may be selected based on, for example, a desired sensitivity level and/or an application in which a method is being practiced. In some embodiments, a ligated template can be directly detected without any amplification, while in other embodiments, ligated template may be amplified such that the copy number of the ligated template is increased, *e.g.*, to enhance sensitivity of a particular assay. Where detection without amplification is practicable, a ligated template may be detected in a number of different ways. For example, oligonucleotide domains of detection probes (*e.g.*, as described and/or utilized herein) may have been directly labeled, *e.g.*, fluorescently or radioisotopically labeled, such that a ligated template is directly labeled. For example, in some embodiments, an oligonucleotide domain of a detection probe (*e.g.*, as provided and/or utilized herein) can comprise a detectable label. A detectable label may be a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Such labels include biotin for staining with labeled Streptavidin conjugate, magnetic beads (*e.g.*, Dynabeads®), fluorescent dyes (*e.g.*, fluorescein, Texas red, rhodamine, green fluorescent protein, and the like), radiolabels (*e.g.*, ^3H , ^{125}I , ^{34}S , ^{14}C , or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and calorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, *etc.*) beads. In some embodiments, a directly labeled ligated template may be size separated from the remainder of the reaction mixture, including unligated directly labeled ligation oligonucleotides, in order to detect the ligated template.

[0498] In some embodiments, detection of a ligated template can include an amplification step, where the copy number of ligated nucleic acids is increased, *e.g.*, in order to enhance sensitivity of the assay. The amplification may be linear or exponential, as desired, where amplification can include, but is not limited to polymerase chain reaction (PCR); quantitative PCR, isothermal amplification, NASBA, digital droplet PCR, *etc.*

[0499] Various technologies for achieving PCR amplification are known in the art; those skilled in the art will be well familiar with a variety of embodiments of PCR technologies, and will readily be able to select those suitable to amplify a ligated template generated using technologies provided herein. For example, in some embodiments, a reaction mixture that includes a ligated template is combined with one or more primers that are employed in the primer extension reaction, *e.g.*, PCR primers (such as forward and reverse primers employed in geometric (or exponential)

amplification or a single primer employed in a linear amplification). Oligonucleotide primers with which one or more ligated templates are contacted should be of sufficient length to provide for hybridization to complementary template DNA under appropriate annealing conditions. Primers are typically at least 10 bp in length, including, *e.g.*, at least 15 bp in length, at least 20 bp in length, at least 25 bp in length, at least 30 bp in length or longer. In some embodiments, the length of primers can typically range from about 15 to 50 bp in length, from about 18 to 30 bp, or about 20 to 35 bp in length. Ligated templates may be contacted with a single primer or a set of two primers (forward and reverse primers), depending on whether primer extension, linear, or exponential amplification of the template DNA is desired.

[0500] In addition to the above components, a reaction mixture comprising a ligated template typically includes a polymerase and deoxyribonucleoside triphosphates (dNTPs). The desired polymerase activity may be provided by one or more distinct polymerase enzymes. In preparing a reaction mixture, *e.g.*, for amplification of a ligated template, various constituent components may be combined in any convenient order. For example, an appropriate buffer may be combined with one or more primers, one or more polymerases and a ligated template to be detected, or all of the various constituent components may be combined at the same time to produce the reaction mixture.

VI. Uses

[0501] In some embodiments, one or more provided biomarkers of one or more target biomarker signatures for ovarian cancer can be detected in a sample comprising biological entities (including, *e.g.*, cells, circulating tumor cells, cell-free DNA, nanoparticles, *etc.*), for example, using methods of detecting and/or assays as described herein. In some embodiments, one or more provided biomarkers of one or more target biomarker signatures for ovarian cancer can be detected in a sample comprising nanoparticles, for example, using methods of detecting and/or assays as described herein.

[0502] In some embodiments, a sample may be or comprise a biological sample. In some embodiments, a biological sample is a bodily fluid sample of a subject (*e.g.*, a human subject). In some embodiments, a biological sample can be derived from a blood or blood-derived sample of a subject (*e.g.*, a human female subject) in need of such an assay. In some embodiments, a biological sample can be or comprise a primary sample (*e.g.*, a tissue or tumor sample) from a subject (*e.g.*, a human female subject) in need of such an assay. In some embodiments, a biological sample can be processed to separate one or more entities of interest (*e.g.*, biological entity) from non-target entities of interest, and/or to enrich one or more entities of interest (*e.g.*, biological entity). In some embodiments, an entity of interest present in a sample may be or comprise a biological entity, *e.g.*, a cell or nanoparticles having a size range that includes extracellular vesicles (*e.g.*, an exosome). In

some embodiments, such a biological entity (*e.g.*, extracellular vesicle) may be processed or contacted with a chemical reagent, *e.g.*, to stabilize and/or crosslink targets (*e.g.*, provided target biomarkers) to be assayed in the biological entity and/or to reduce non-specific binding with detection probes. In some embodiments, 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 embodiments, 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.

[0503] In some embodiments, technologies provided herein can be useful for managing patient care, *e.g.*, for one or more individual subjects and/or across a population of subjects. By way of example only, in some embodiments, provided technologies may be utilized in screening, which for example, may be performed periodically, such as annually, semi-annually, bi-annually, or with some other frequency as deemed to be appropriate by those skilled in the art. In some embodiments, such a screening may be temporally motivated or incidentally motivated. For example, in some embodiments, provided technologies may be utilized in temporally motivated screening for one or more individual subjects or across a population of subjects (*e.g.*, asymptomatic female subjects) who are older than a certain age (*e.g.*, over 40, 45, 50, 55, 60, 65, 70, 75, 80, or older). As will be appreciated by those skilled in the art, in some embodiments, the screening age and/or frequency may be determined based on, for example, but not limited to prevalence of a disease, disorder, or condition (*e.g.*, cancer such as ovarian cancer). In some embodiments, provided technologies may be utilized in incidentally motivated screening for individual subjects who may have experienced an incident or event that motivates screening for a particular disease, disorder, or condition (*e.g.*, cancer such as ovarian cancer). For example, in some embodiments, an incidental motivation relating to determination of one or more indicators of a disease, disorder, or condition (*e.g.*, cancer such as ovarian 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 such a disease, disorder, or condition such as ovarian cancer or breast cancer), identification of one or more life-history associated risk factors for a disease, disorder, or condition (*e.g.*, ovarian cancer) 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 a particular disease, disorder, or condition (*e.g.*, abnormal bleeding during a woman's period potentially indicative of ovarian cancer, *etc.*) and/or other incidents or events as will be appreciated by those skilled in the art.

[0504] In some embodiments, 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 embodiments, provided technologies can provide determination of whether individual subjects have one or more indicators of risk, incidence, or recurrence of a disease disorder, or condition (*e.g.*, cancer such as ovarian cancer), thereby informing physicians and/or patients when to provide/receive therapeutic or prophylactic recommendations and/or to initiate such therapy in light of such findings. In some embodiments, such individual subjects may be asymptomatic subjects, who may be temporally motivated or incidentally-motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such individual subjects may be experiencing one or more symptoms that may be associated with ovarian cancer, who may be temporally motivated or incidentally motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such individual subjects may be subjects having a benign gynecological tumor and/or a chronic inflammatory condition, who may be temporally motivated or incidentally motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such individual subjects may be subjects at hereditary risk for ovarian cancer, who may be temporally motivated or incidentally-motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such individual subjects may be subjects with life-history associated risk, who may be temporally motivated or incidentally-motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such individual subjects may be post-menopausal subjects, who may be temporally motivated or incidentally motivated to be screened at a regular frequency (*e.g.*, annually, semi-annually, bi-annually, or other frequency as deemed to be appropriate by those skilled in the art). In some embodiments, such post-menopausal subjects may be experiencing abdominal pain and/or pelvic pain.

[0505] Additionally or alternatively, in some embodiments, 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 embodiments, 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, thereby informing physicians and/or patients of efficacy of such therapy and/or decisions to maintain or alter therapy in light of such findings. In some embodiments, provided technologies can provide determination of whether individual subjects are likely to be responsive to a recommended treatment, *e.g.*, based on findings of molecular targets (*e.g.*, provided biomarkers of one or more target biomarker signatures for ovarian cancer) that predict therapeutic

effects of a recommended treatment on individual subjects, thereby informing physicians and/or patients of potential efficacy of such therapy and/or decisions to administer or alter therapy in light of such findings.

[0506] In some embodiments, 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 embodiments, the present disclosure provides methods relating to (a) receiving results of a screening that employs provided technologies 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 (based on, *e.g.*, screening age such as older than a certain age, *e.g.*, over 40, 45, 50, 55, 60, 65, 70, 75, 80, or older, and/or screening frequency such as, *e.g.*, every 3 months, every 6 months, every year, every 2 years, every 3 years or at some other frequencies) or in 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 embodiments, 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).

[0507] 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 (*e.g.*, as generated in accordance with the present disclosure), and/or of reimbursement decisions as described herein. Various reporting systems are known in the art; those skilled in the art will be well familiar with a variety of such embodiments, and will readily be able to select those suitable for implementation.

Exemplary uses

A. Detection of ovarian cancer incidence or recurrence

[0508] The present disclosure, among other things, recognizes that detection of a single cancer-associated biomarker in a biological entity (*e.g.*, extracellular vesicle) or a plurality of cancer-associated biomarkers based on a bulk sample, rather than at a resolution of a single biological entity (*e.g.*, individual nanoparticles, including, *e.g.*, extracellular vesicles), typically does not provide sufficient specificity and/or sensitivity in determination of whether a subject from whom the biological entity is obtained is likely to be suffering from or susceptible to cancer (*e.g.*, ovarian

cancer). The present disclosure, among other things, provides technologies, including compositions and/or methods, that solve such problems, including for example by specifically requiring that an entity (*e.g.*, an extracellular vesicle) for detection be characterized by presence of a combination of at least two or more targets (*e.g.*, at least two or more provided biomarkers of a target biomarker signature for ovarian cancer). In particular embodiments, the present disclosure teaches technologies that require such an entity (*e.g.*, an extracellular vesicle) be characterized by presence (*e.g.*, by expression) of a combination of molecular targets that is specific to cancer (*i.e.*, “target biomarker signature” of a relevant cancer, *e.g.*, ovarian cancer), while biological entities (*e.g.*, nanoparticles, including, *e.g.*, extracellular vesicles) that do not comprise the targeted combination (*e.g.*, target biomarker signature) do not produce a detectable signal. Accordingly, in some embodiments, technologies provided herein can be useful for detection of risk, incidence, and/or recurrence of cancer in a subject. In some such embodiments, technologies provided herein are useful for detection of risk, incidence, and/or recurrence of ovarian cancer in a female subject. For example, in some embodiments, a combination of two or more provided biomarkers are selected for detection of a specific cancer (*e.g.*, ovarian cancer) or various cancers (one of which includes ovarian cancer). In some embodiments, a specific combination of provided biomarkers for detection of ovarian cancer can be determined by analyzing a population or library (*e.g.*, tens, hundreds, thousands, tens of thousands, hundreds of thousands, or more) of ovarian cancer patient biopsies and/or patient data to identify such a predictive combination. In some embodiments, a relevant combination of biomarkers may be one identified and/or characterized, for example, via data analysis. For example, in some embodiments, data analysis may comprise a bioinformatic analysis. In some embodiments, for example, a diverse set of ovarian cancer-associated data (*e.g.*, in some embodiments comprising one or more of bulk RNA sequencing, single-cell RNA (scRNA) sequencing, mass spectrometry, histology, post-translational modification data, *in vitro* and/or *in vivo* experimental data) can be analyzed through machine learning and/or computational modeling to identify a combination of predictive markers that is highly specific to ovarian cancer. In some embodiments, a combination of predictive markers to distinguish stages of cancer (*e.g.*, ovarian cancer) can be determined *in silico* based on comparing and analyzing diverse data (*e.g.*, in some embodiments comprising bulk RNA sequencing, scRNA sequencing, mass spectrometry, histology, post-translational modification data, *in vitro* and/or *in vivo* experimental data) relating to different stages of cancer (*e.g.*, ovarian cancer). For example, in some embodiments, technologies provided herein can be used to distinguish ovarian cancer subjects from non-ovarian cancer subjects, including, *e.g.*, healthy female subjects, female subjects diagnosed with benign tumors or adnexal masses, and female subjects with non-ovarian-related diseases, disorders, and/or conditions (*e.g.*, female subjects with non-ovarian cancer, or subjects with inflammatory conditions, *e.g.*, Crohn’s disease, ulcerative colitis). In some embodiments, technologies provided herein can be useful for early detection of ovarian cancer, *e.g.*,

detection of ovarian cancer of stage I or stage II. For example, in some embodiments, a set of biomarker combinations as shown in **Table 5** or **Table 8** can be particularly useful for detection of early-stage ovarian cancer. In some embodiments, technologies provided herein can be useful for detection of one or more ovarian cancer subtypes, including, *e.g.*, high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer. In some embodiments, technologies provided herein can be useful for screening individuals at hereditary risk, life-history associated risk, or average risk for early stage high-grade serous ovarian cancer (HGSOC).

[0509] In some embodiments, technologies provided herein can be useful for screening a subject for risk, incidence, or recurrence of a specific cancer in a single assay. For example, in some embodiments, technologies provided herein is useful for screening a subject for risk, incidence, or recurrence of ovarian cancer. In some embodiments, technologies provided herein can be used to screen a subject for risk or incidence of a specific cancer or a plurality of (*e.g.*, at least 2, at least 3, or more) cancers in a single assay. For example, in some embodiments, technologies provided herein can be used to screen a subject for a plurality of cancers in a single assay, one of which includes ovarian cancer and other cancers to be screened can be selected from the group consisting of brain cancer (including, *e.g.*, glioblastoma), breast cancer, colorectal cancer, pancreatic cancer, prostate cancer, liver cancer, lung cancer, and skin cancer.

[0510] In some embodiments, provided technologies can be used periodically (*e.g.*, every year, every two years, every three years, *etc.*) to screen a human subject for ovarian cancer (*e.g.*, early-stage ovarian cancer) or cancer recurrence. In some embodiments, a human subject amenable to such screening may be an adult or an elderly. In some embodiments, a human subject amenable to such screening may be a post-menopausal woman. In some embodiments, a human subject amenable to such screening may be older than a specified age, *e.g.*, age 45 and above, age 55 and above, age 65 and above, age 70 and above, age 75 above, or age 80 and above. In some embodiments, a human subject amenable to such screening may have an age of about 50 or above. In some embodiments, a human subject amenable to such screening may have an age of 50 or less. In some embodiments, a human subject amenable to such screening may have an age over 35. In some embodiments, a human subject who is determined to have a genetic predisposition to ovarian cancer may be screened at a younger age than a human subject who has no family history risk.

[0511] In some embodiments, a subject that is amenable to provided technologies for detection of incidence or recurrence of ovarian cancer may be a post-menopausal human female subject, who in some embodiments may be experiencing abdominal pain and/or pelvic pain. In some embodiments, a subject that is amenable to provided technologies for detection of incidence or recurrence of ovarian cancer may be a human female subject who is at least 55 years old and is determined to have a benign gynecological tumor and/or one or more chronic inflammatory

conditions. In some embodiments, a subject that is amenable to provided technologies for detection of incidence or recurrence of ovarian cancer may be a post-menopausal human female subject or a human female subject at age of least 55 years old, who has a family history of breast and/or ovarian cancer (*e.g.*, women having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer), who has been previously treated for cancer (*e.g.*, ovarian cancer), who is at risk of ovarian cancer recurrence after cancer treatment, who is in remission after ovarian cancer treatment, and/or who has been previously or periodically screened for ovarian cancer, *e.g.*, by screening for the presence of at least one ovarian cancer biomarker (*e.g.*, by detecting serum protein CA-125 and/or by transvaginal ultrasound (TVUS)). Alternatively, in some embodiments, a post-menopausal human female subject or a human female subject at age of least 55 years old may be a female subject who has not been previously screened for ovarian cancer, who has not been diagnosed for ovarian cancer, and/or who has not previously received ovarian cancer therapy. In some embodiments, a post-menopausal human female subject or a human female subject at age of least 55 years old may be a female subject with a benign gynecological tumor. In some embodiments, a post-menopausal subject may be a subject who is susceptible to ovarian cancer (*e.g.*, at an average population risk, at increased risk due to life-history factors, at increased risk due to menopause, or with hereditary risk for ovarian cancer).

[0512] In some embodiments, the present disclosure, among other things, provides insights that technologies described and/or utilized herein may be particularly useful for screening certain populations of female subjects, *e.g.*, female subjects who are at higher susceptibility to developing ovarian cancer. In some embodiments, the present disclosure, among other things, recognizes that the resulting PPVs of technologies described and/or utilized herein for HGSOV detection may be higher in ovarian cancer prone or susceptible populations. In some embodiments, the present disclosure, among other things, provides insights that screening of post-menopausal 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 ovarian cancer. In some embodiments, the present disclosure provides ovarian cancer screening systems that can be implemented to detect ovarian cancer, including early-stage cancer, in some embodiments in post-menopausal individuals (*e.g.*, with or without hereditary and/or life-history risks in ovarian cancer and/or with or without symptoms such as abdominal and/or pelvic pain). In some embodiments, provided technologies can be implemented to achieve regular screening of post-menopausal individuals (*e.g.*, with or without hereditary and/or life-history risks in ovarian cancer and/or with or without symptoms such as abdominal and/or pelvic pain). In some embodiments, provided technologies achieve detection (*e.g.*, early detection, *e.g.*, in symptomatic or asymptomatic individual(s) and/or population(s)) of one or more features (*e.g.*, incidence, progression, responsiveness to therapy, recurrence, *etc.*) of ovarian cancer, 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 embodiments, provided technologies are useful in conjunction with a subject's periodic physical examination such as mammograms, HPV, and/or Pap smear screening (*e.g.*, every year, every other year, or at an interval approved by the attending physician). In some embodiments, provided technologies are useful in conjunction with treatment regimen(s); in some embodiments, provided technologies may improve one or more characteristics (*e.g.*, rate of success according to an accepted parameter) of such treatment regimen(s).

[0513] In some embodiments, a subject that is amenable to provided technologies for detection of incidence or recurrence of ovarian cancer may be an asymptomatic human female subject and/or across an asymptomatic population of female subjects. Such an asymptomatic subject and/or across an asymptomatic population of female subjects may be subject(s) who has/have a family history of breast cancer and/or ovarian cancer (*e.g.*, women having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer), who has been previously treated for cancer (*e.g.*, ovarian cancer), who is at risk of ovarian cancer recurrence after cancer treatment, who is in remission after ovarian cancer treatment, and/or who has been previously or periodically screened for ovarian cancer, *e.g.*, by screening for the presence of at least one ovarian cancer biomarker (*e.g.*, by detecting serum protein CA-125 and/or by transvaginal ultrasound (TVUS)). Alternatively, in some embodiments, an asymptomatic subject may be a female subject who has not been previously screened for ovarian cancer, who has not been diagnosed for ovarian cancer, and/or who has not previously received ovarian cancer therapy. In some embodiments, an asymptomatic subject may be a female subject with a benign gynecological tumor. In some embodiments, an asymptomatic subject may be a subject who is susceptible to ovarian cancer (*e.g.*, at an average population risk or with hereditary risk for ovarian cancer).

[0514] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be selected based on one or more characteristics such as age, race, geographic location, 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). For example, in some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) determined to have one or more germline mutations in ovarian cancer-associated genes, including but not limited to, *e.g.*, BARD1, BRIP1, RAD51C, RAD51D, CHEK2, MRE11A, RAD50, ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11, TP53, and combinations thereof. In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer

may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) determined to have one or more germline single nucleotide polymorphisms at specific loci or within certain genes determined by genome wide association studies to be associated with ovarian-cancer, including but not limited to *e.g.*, WNT4, RSP01, BCL2L11, HOXD3, HAGLR, TIPARP, SYNPO2, TERT, GPX6, CHMP4C, LINC00824, COL15A1, SMC2-AS1, MLLT10, INCENP, RCCD1, ATAD5, HNF1B, PLEKHM1, SKAP1, ANKLE1, GATAD2A, Cytobands and SNPs 2q13 rs752590, 4q32.3 rs4691139, 9p22 rs3814113, 9q34.2 rs635634, 10p11.21 rs1192691, and/or 19q13.2 rs688187 (Reid *et al.*, 2017; which is incorporated herein by reference for the purpose described herein).

[0515] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) with breast cancer determined to have germline mutations in BRCA1, BRCA2 and/or PALB2.

[0516] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be selected based on one or more characteristics such as age, race, geographic location, genetic history, medical history, personal history (*e.g.*, smoking, alcohol, drugs, carcinogenic agents, diet, obesity, diabetes, nulliparousness/infertility, no history/short history of oral contraceptive use, physical activity, sun exposure, radiation exposure, perineal talc use, hormone replacement therapy (HRT), exposure to infectious agents such as viruses, and/or occupational hazard). For example, in some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) determined to have one or more life-history associated risk factors.

[0517] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) diagnosed with an imaging-confirmed adnexal mass or pelvic mass.

[0518] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) at hereditary risk before undergoing a biopsy and/or a surgical procedure (*e.g.*, bilateral salpingo-oophorectomy).

[0519] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) with one or more non-specific symptoms of ovarian cancer. In some embodiments, exemplary non-specific symptoms of ovarian cancer may include symptoms similar to one or more symptoms for irritable bowel syndrome.

[0520] In some embodiments, a subject that is amenable to provided technologies for detection of incidence or recurrence of ovarian cancer may be a symptomatic human female subject and/or across a symptomatic population of female subjects. Such a symptomatic subject and/or across a symptomatic population of female subjects may be subject(s) who has/have a family history of breast and/or ovarian cancer (*e.g.*, women having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer), who has been previously treated for cancer (*e.g.*, ovarian cancer), who is at risk of ovarian cancer recurrence after cancer treatment, who is in remission after ovarian cancer treatment, and/or who has been previously or periodically screened for ovarian cancer, *e.g.*, by screening for the presence of at least one ovarian cancer biomarker (*e.g.*, by detecting serum protein CA-125 and/or by transvaginal ultrasound (TVUS)). Alternatively, in some embodiments, a symptomatic subject may be a female subject who has not been previously screened for ovarian cancer, who has not been diagnosed for ovarian cancer, and/or who has not previously received ovarian cancer therapy. In some embodiments, a symptomatic subject may be a female subject with a benign gynecological tumor. In some embodiments, a symptomatic subject may be a subject who is susceptible to ovarian cancer (*e.g.*, at an average population risk, with hereditary risk for ovarian cancer, with life-history associated risk for ovarian cancer, and/or with age associated risk for ovarian cancer).

[0521] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) of diverse descent such as Asians, African Americans, Caucasians, Native Hawaiians or other Pacific Islanders, Hispanics or Latinos, American Indians or Alaska natives, non-Hispanic blacks, or non-Hispanic whites. In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) of diverse descent such as Asian Pacific Islanders, Hispanics, American Indian/Alaska natives, non-Hispanic black, or non-Hispanic whites. In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be a female subject (*e.g.*, a woman) or a population of female subjects (*e.g.*, women) of any race and/or any ethnicity.

[0522] In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be determined to have a normal plasma CA-125 level (*e.g.*, a level of less than 35 U/mL). In some embodiments, a subject or population of subjects that are amenable to provided technologies for detection of ovarian cancer may be determined to have a plasma CA-125 level that is equal to or higher than a normal plasma CA-125 level (*e.g.*, a level of less than 35 U/mL).

[0523] In some embodiments, technologies provided herein can be used in combination with other diagnostics assays including, *e.g.*, but not limited to (i) a woman's annual physical

examination (*e.g.*, including a HPV, and/or Pap smear screening for cervical cancer and a mammogram screening for breast cancer); (ii) plasma CA-125 and/or TVUS 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 immunofluorescence staining to identify cell phenotype and marker expression, followed by amplification and analysis by next-generation sequencing; and (v) BRCA1 and/or BRCA2 germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, OVA1® and OVERA® tests, and/or circulating tumor cells.

B. Selection of cancer therapy (e.g., ovarian cancer therapy)

[0524] In some embodiments, provided technologies can be used for selecting an appropriate treatment for a cancer patient (*e.g.*, a patient suffering from or susceptible to ovarian cancer). For example, some embodiments provided herein relate to a companion diagnostic assay for classification of patients for cancer therapy (*e.g.*, ovarian cancer and/or adjunct treatment) which comprises assessment in a patient sample (*e.g.*, a biological sample such as a blood-derived sample from an ovarian cancer patient) of a selected combination of provided biomarkers using technologies provided herein. Based on such an assay outcome, patients who are determined to be more likely to respond to a cancer therapy (*e.g.*, an ovarian cancer therapy and/or an adjunct therapy, including, *e.g.*, olaparib, cisplatin, rucaparib, niraparib, talazoparib) can be administered such a therapy, or patients who are determined to be non-responsive to a specific such therapy can be administered a different therapy.

C. Evaluation of treatment efficacy (e.g., cancer treatment efficacy)

[0525] In some embodiments, technologies provided herein can be used for monitoring and/or evaluating efficacy of an anti-cancer therapy administered to a cancer patient (*e.g.*, ovarian cancer patient). For example, a biological sample (*e.g.*, a bodily fluid sample including, but not limited to a blood-derived sample, etc.) can be collected from an ovarian cancer patient prior to or receiving an anti-cancer therapy (*e.g.*, olaparib, cisplatin, rucaparib, niraparib, talazoparib) at a first time point to detect or measure tumor burdens, *e.g.*, by detecting presence or amount of nanoparticles comprising a selected combination of biomarkers that is specific to detection of ovarian cancer. After a period of treatment, a second biological sample (*e.g.*, a bodily fluid sample including, but not limited to a blood-derived sample, etc.) can be collected from the same ovarian cancer patient to detect changes in tumor burdens, *e.g.*, by detecting absence or reduction in amount of nanoparticles comprising a selected combination of biomarkers that is specific to detection of ovarian cancer. By monitoring levels and/or changes in tumor burdens over the course of treatment, appropriate course of action, *e.g.*, increasing or decreasing the dose of a therapeutic agent, and/or administering a

different therapeutic agent, can be taken.

D. Differentiation of a benign adnexal mass from ovarian cancer

[0526] In some embodiments, provided technologies are particularly useful to aid in the diagnosis of ovarian cancer in symptomatic individuals with an imaging-confirmed adnexal mass. Adnexal masses (*i.e.*, a growth or lump in tissue near the uterus) are commonly detected in women, often with imaging methods (*e.g.*, with transvaginal ultrasound). It is estimated that around 5-10% of women will undergo surgical removal of an adnexal mass at some point during their lifetime (*see*, for example, Piovano *et al.*, "Diagnostic accuracy and cost-effectiveness of different strategies to triage women with adnexal masses: a prospective study." *Ultrasound in Obstetrics & Gynecology* 50.3 (2017)). Adnexal masses can be the result of various underlying pathologies, which may include, for example, ovarian cysts, ectopic pregnancies, benign tumors, or malignant cancers. Therefore, further testing and/or screening is required beyond imaging methods so that the underlying pathology of an adnexal mass can be properly diagnosed.

[0527] It is estimated that around 200,000 women are detected to have an adnexal mass in the United States per year, and about 45% of these cases do not provide any conclusive results as to whether the adnexal mass is cancer or benign based on image interpretation. There are currently multiple diagnostic tests available to follow up on an adnexal mass detection so as to determine if the adnexal mass is cancerous or benign. These follow up diagnostic tests may include, for example, clinical assessment (CA), transvaginal (TV) ultrasound, CA-125, CA-125 & CA, OVA1, OVERA, OVERA & CA, and ROMA (HE4 & CA-125).

[0528] A clinical assessment of a patient detected to have an adnexal mass can involve, for example, a clinician assessing a patient's age, menopausal status, personal and/or familial history of breast and/or ovarian cancer, genotype (*e.g.*, BRCA1 and/or BRCA2), symptoms of pelvic pain, abdominal distension, gastrointestinal complaints, weight loss, personal history of hormonal contraception, and/or other parameters (*see*, for example, Smorgick *et al.*, "Assessment of adnexal masses using ultrasound: a practical review." *International journal of women's health* 6 (2014)).

[0529] Transvaginal ultrasound (TVUS) typically involves a trained clinician qualitatively evaluating various morphological parameters in ultrasound images (*e.g.* septation thickness, cyst wall thickness, solid components, etc.) to diagnose malignancy (*see*, for example, Smorgick *et al.*, "Assessment of adnexal masses using ultrasound: a practical review." *International journal of women's health* 6 (2014)).

[0530] CA-125 (or CA125), otherwise known as cancer antigen 125, is a protein that may be elevated in the blood of patients with certain types of cancer (*e.g.*, ovarian cancer). CA-125 can be measured by methods known in the art (*e.g.*, by ELISA) to assess the amount of CA-125 (*e.g.*, units per mL) in a blood sample.

[0531] OVA1 is a first-generation multivariate index assay (MIA) that evaluates the levels of five ovarian cancer-associated markers in a blood sample and utilizes a mathematical formula to combine these levels into a single ovarian cancer risk score. OVA1 received FDA approval in 2008 for the detection of ovarian cancer risk in women planned for surgery for an adnexal mass.

[0532] OVERA is a second-generation MIA that incorporates additional markers to OVA1 and employs an adjusted algorithm. OVERA received FDA approval in 2016 for the detection of ovarian cancer risk in women planned for surgery for an adnexal mass.

[0533] ROMA is an algorithm-based test that derives a numerical score from the results of serum CA-125 and HE4 levels plus menopausal status to identify patients presenting with an adnexal mass as being at high or low likelihood of having malignancy. Similar to CA-125, HE4 is a protein that can be measured in a blood sample and may be elevated in certain cancer types. ROMA received FDA approval in 2011 to determine the likelihood of malignancy in premenopausal or postmenopausal women presenting with an adnexal mass.

[0534] The present disclosure appreciates that many of such conventional diagnostic assays can be time-consuming, costly, and/or lacking sensitivity and/or specificity sufficient to provide a reliable and comprehensive diagnostic assessment. **Table 2** below shows the sensitivity, specificity, and predictive values of certain conventional diagnostic tests.

Table 2. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of certain convention diagnostic tests for the diagnosis of an adnexal mass as cancerous.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Citation
Clinical Assessment (CA)	74.5	86.3	64.6	91.0	Longoria, 2014
TV Ultrasound (MI>5)	73.3	74.7	26.2	95.7	Pavlik, 2009
CA-125 Alone	70.6	89.6	69.5	90.1	Longoria, 2014
CA-125 & CA (ACOG)	77.0	68.0	52.0	87.0	Miller, 2012
OVA1 Alone	92.4	53.5	31.3	96.8	Bristow, 2016
OVA1 & CA	95.7	50.7	30.8	98.1	Bristow, 2016
OVERA Alone	91.3	69.1	40.4	97.2	FDA K150588, 2016
OVERA & CA	93.5	64.8	37.9	97.7	FDA K150588, 2016
ROMA (HE4 & CA-125)	80.9	74.9	42.9	94.4	Moore <i>et al.</i> , 2011

[0535] In some embodiments, the present disclosure provides technologies (including systems, compositions (including biomarker combinations), and methods) that solve such problems,

among other things, by detecting co-localization of at least one biomarker combination described herein, which comprises at least one capture biomarker (*e.g.*, a surface biomarker described herein selected as a target of a capture probe described herein, *e.g.*, to capture nanoparticles from a sample described herein) and at least one detection biomarker (*e.g.*, a surface biomarker described herein selected as a target of a detection probe described herein), on surfaces of individual intact nanoparticles. In some embodiments, the present disclosure, among other things, identifies biomarker combinations described herein (*e.g.*, in some embodiments biomarker combinations as shown in **Table 5** or **Table 8**) and provides assays that utilize such biomarker combination(s) describes herein that can be particularly useful for differentiating benign adnexal mass from ovarian cancer with increased sensitivity, specificity, PPV, and/or NPV as compared to the conventional diagnostic tests as listed in **Table 2**. In some embodiments, such assays described herein have a sensitivity of 95% or greater in differentiating a benign adnexal mass from ovarian cancer. In some embodiments, assays described herein have a specificity of 90% or greater in differentiating a benign adnexal mass from ovarian cancer. In some embodiments, assays described herein have a PPV of 70% or greater in differentiating a benign adnexal mass from ovarian cancer. In some embodiments, assays described herein have a NPV of 98% or greater in differentiating a benign adnexal mass from ovarian cancer.

[0536] In some embodiments, a positive test result from assays that utilize one or more biomarker combinations described herein (*e.g.*, in some embodiments biomarker combinations as shown in **Table 5** or **Table 8**) can be interpreted in conjunction with other clinical findings to diagnose cancer. In some embodiments, such clinical findings to diagnose cancer may include, for example, pelvic or abdominal pain, inability to eat or feeling "full," and/or increased abdominal size or bloating, and other clinical findings as described, for example, for example, in Goff *et al.*, Development of an ovarian cancer symptom index. *Cancer*. 2007;109:221-227, the entire content of which is incorporated herein by reference for the purposes described herein.

VII. Kits

[0537] Also provided are kits that find use in practicing technologies as described above. In some embodiments, a kit comprises a plurality of detection probes (*e.g.*, as described and/or utilized herein). In some embodiments, a provided kit 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 embodiments, individual detection probes may be directed at different targets. In some embodiments, two or more individual detection probes may be directed to the same target. In some embodiments, a provided 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. In some embodiments, a provided kit comprises a plurality of subsets of detection probes,

each of which comprises two or more detection probes directed at the same target. In some embodiments, a plurality of detection probes may be provided as a mixture in a container. In some embodiments, multiple subsets of detection probes may be provided as individual mixtures in separate containers. In some embodiments, each detection probe is provided individually in a separate container.

[0538] In some embodiments, a kit that is useful for methods and/or uses described herein comprises at least one set of probes for a biomarker combination specific for detection of ovarian cancer, wherein the biomarker combination comprises at least one capture biomarker on exosomes and at least one detection biomarker on exosomes, and wherein the capture biomarker and the detection biomarker are each independently selected from: (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and (iii) combinations thereof; and wherein the at least one set of probes comprises: a capture probe comprising a target-capture moiety directed to the capture biomarker; and at least two detection probes each comprising a target binding moiety directed to the at least one detection biomarker. In some embodiments, a capture biomarker is a biomarker of a biomarker combination described herein (*e.g.*, in some embodiments, a biomarker of a biomarker combination as shown in **Table 1**, **Table 5**, or **Table 8**). In some embodiments, a detection biomarker is a biomarker of a biomarker combination described herein (*e.g.*, in some embodiments, a biomarker of a biomarker combination as shown in **Table 1**, **Table 5**, or **Table 8**). In some embodiments, such a kit comprises a plurality of sets of probes described herein, each set for a distinct biomarker combination specific for detection of ovarian cancer. For example, in some embodiments, one or more biomarker combination(s) for inclusion in a set can be selected from one of the following:

- i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0539] In some embodiments, a kit that is useful for methods and/or uses described herein comprises at least seven sets of probes, each set for a distinct biomarker combination as follows:

- i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

[0540] In some embodiments, capture probe(s) and detection probes provided in a kit described herein selectively bind to respective biomarkers on exosomes with a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95% or within a range of 70% to 95%, or within a range of 75% to 95%, or within a range of 80% to 95%. In some such embodiments, detection probes provided in a kit described herein each comprises a target binding moiety directed to at least one detection biomarker on exosomes (*e.g.*, in some embodiments, a biomarker of a biomarker combination as shown in *Table 1*, *Table 5*, or *Table 8*); and 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same exosome.

[0541] In some embodiments, a kit for detection of ovarian cancer comprises: (a) a capture agent comprising a target-capture moiety directed to an extracellular vesicle-associated surface biomarker; and (b) a set of detection probes, which set comprises at least two detection probes each directed to a target biomarker of a target biomarker signature for ovarian cancer, wherein the detection probes each comprise: (i) a target binding moiety directed the target biomarker of the target biomarker signature for ovarian cancer; 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 at least two detection probes are characterized in that they can

hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle.

[0542] In some embodiments, the present disclosure describes a kit for detection of ovarian cancer comprising: (a) a capture agent comprising a target-capture moiety directed to a first surface biomarker; and (b) at least one set of detection probes, which set comprises at least two detection probes each directed to a second surface biomarker, wherein the detection probes each comprise: (i) a target binding moiety directed at the second surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same nanoparticle having the size within the range of about 30 nm to about 1000 nm; wherein at least the first surface biomarker and the second surface biomarker form a target biomarker signature determined to be associated with ovarian cancer, and wherein the first and second surface biomarkers are each independently selected from: (A) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (B) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0543] In many embodiments described herein, a target biomarker signature for ovarian cancer comprises: at least one extracellular vesicle-associated surface biomarker and at least one target biomarker comprising at least one surface biomarker, wherein: the extracellular vesicle-associated surface biomarker and the target biomarker are each independently selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0544] In some embodiments, a kit for detection of ovarian cancer further comprises at least one intravesicular protein biomarker and/or at least one intravesicular RNA biomarker. In some embodiments, an exemplary intravesicular protein biomarker is or comprises CRABP2, KLK7, MIF, PRAME, and S100A1, and combinations thereof. In some embodiments, an exemplary intravesicular RNA biomarker is or comprises CRABP2, MIF, CLDN6, PRAME, S100A1, KLK7, and combinations thereof.

[0545] In some embodiments, when at least one target biomarker is selected from one or more of the provided surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different. In some embodiments, when at least

one target biomarker is selected from one or more of the provided surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are the same (with the same or different epitopes).

[0546] In some embodiments, a capture agent provided in a kit comprises a target-capture moiety directed to an extracellular vesicle-associated surface biomarker, which is or comprises (i) a polypeptide encoded by a human gene as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), or combinations thereof.

[0547] In some embodiments, a target binding moiety of at least two detection probes provided in a kit is each directed to the same target biomarker of a target biomarker signature. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *FOLR1* and *FOLR1*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *MUC16* and *MUC16*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BST2* and *BST2*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *CLDN3* and *CLDN3*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *SLC34A2* and *SLC34A2*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BCAM* and *BCAM*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to sTn antigen and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *MUC1* and *MUC1*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *MUC16* and cleaved *MUC16*, respectively. In some such embodiments, an oligonucleotide domain of such at least two detection probes are different.

[0548] In some embodiments, a target binding moiety of at least two detection probes provided in a kit is each directed to a distinct target biomarker of a target biomarker signature. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *FOLR1* and *MUC16*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BST2* and *MUC16*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BCAM* and *BST2*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BST2* and *FOLR1*, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to *BST2* and *MUC1*, respectively. In certain embodiments, at least two detection probes in a kit may have their

target binding entities directed to BST2 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MSLN and MUC1, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to FOLR1 and SLC34A2, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MUC1 and MUC16, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MUC1 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MUC16 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to SLC34A2 and sTn antigen, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to FOLR1 and MSLN, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MUC16 and MSLN, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to FOLR1 and MUC1, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to MUC1 and SLC34A2, respectively. In certain embodiments, at least two detection probes in a kit may have their target binding entities directed to cleaved MUC16 and MSLN, respectively.

[0549] In certain embodiments, wherein a target biomarker signature comprises a combination of SLC34A2 and FOLR1, a capture probe has their target binding entity directed to SLC34A2 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of SLC34A2 and MUC16, a capture probe has their target binding entity directed to SLC34A2 and at least two detection probes have their target binding entities directed to MUC16 and MUC16, respectively.

[0550] In certain embodiments, wherein a target biomarker signature comprises a combination of BST2 and FOLR1, a capture probe has their target binding entity directed to BST2 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively.

[0551] In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and BST2, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and BST2 and MUC16, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities

directed to BST2 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and CLDN3, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to CLDN3 and CLDN3, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of CA19-9 antigen and SLC34A2, a capture probe has their target binding entity directed to CA19-9 antigen and at least two detection probes have their target binding entities directed to SLC34A2 and SLC34A2, respectively.

[0552] In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BCAM, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BCAM and BCAM, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BCAM and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BCAM and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2 and FOLR1, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and BST2 and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to BST2 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and MSLN, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to MSLN and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and MSLN and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC1 and sTn antigen, a capture probe has their target binding entity directed to MUC1 and at least two detection probes have their target binding entities directed to sTn antigen and sTn antigen, respectively.

[0553] In certain embodiments wherein a target biomarker signature comprises a combination of MUC16 and FOLR1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and BCAM, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to BCAM and BCAM, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and FOLR1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and FOLR1 and SLC34A2, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to FOLR1 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MUC1 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC1 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MUC16 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and MSLN and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to MSLN and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and SLC34A2, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to SLC34A2 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and SLC34A2 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two detection probes have their target binding entities directed to SLC34A2 and sTn antigen, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of MUC16 and sTn antigen, a capture probe has their target binding entity directed to MUC16 and at least two

detection probes have their target binding entities directed to sTn antigen and sTn antigen, respectively.

[0554] In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and BST2 and MUC1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to BST2 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MUC16, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC1 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC1 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC16 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC16 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and MUC1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and MUC1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and FOLR1, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC1 and SLC34A2, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC1 and SLC34A2, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and MUC16 and cleaved MUC16, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to MUC16 and cleaved MUC16, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and cleaved MUC16 and MSLN, a capture probe has their target binding entity directed to sTn antigen and at least two detection probes have their target binding entities directed to cleaved MUC16 and MSLN, respectively. In certain embodiments, wherein a target biomarker signature comprises a combination of sTn antigen and FOLR1 and SLC34A2, a capture probe has their target binding entity directed to

sTn antigen and at least two detection probes have their target binding entities directed to FOLR1 and SLC34A2, respectively.

[0555] In certain embodiments, wherein a target biomarker signature comprises a combination of T antigen and BST2, a capture probe has their target binding entity directed to T antigen and at least two detection probes have their target binding entities directed to BST2 and BST2, respectively.

[0556] In some embodiments, a target binding moiety of a capture probe and/or a detection probe may be or comprise an affinity agent, which in some embodiments may be or comprise an antibody (*e.g.*, a monoclonal antibody). In some embodiments, a target binding moiety of a detection probe may be or comprise an affinity agent, which in some embodiments may be or comprise a lectin or siglec.

[0557] In some embodiments, a kit may comprise at least one chemical reagent such as a fixation agent, a permeabilization agent, and/or a blocking agent.

[0558] In some embodiments, a kit may comprise one or more nucleic acid ligation reagents (*e.g.*, a nucleic acid ligase such as a DNA ligase and/or a buffer solution).

[0559] In some embodiments, a kit may comprise at least one or more amplification reagents such as PCR amplification reagents. In some embodiments, a kit may comprise one or more nucleic acid polymerases (*e.g.*, DNA polymerases), one or more pairs of primers, nucleotides, and/or a buffered solution.

[0560] In some embodiments, a kit may provide reagents and/or materials for purification of an entity (*e.g.*, biological entity) of interest from a subject's sample. In some embodiments, a kit may provide reagents and/or materials for purification of nanoparticles from a patient's sample (*e.g.*, plasma sample), *e.g.*, in some embodiments by size-exclusion chromatography. For example, in some embodiments, a kit may comprise a solid substrate for capturing an entity (*e.g.*, biological entity) of interest. For example, such a solid substrate may be or comprise a bead (*e.g.*, a magnetic bead). In some embodiments, such a solid substrate may be or comprise a surface. In some embodiments, a surface may be or comprise a capture surface (*e.g.*, an entity capture surface) of an assay chamber, such as, *e.g.*, a filter, a matrix, a membrane, a plate, a tube, a well (*e.g.*, but not limited to a microwell), *etc.* In some embodiments, a surface (*e.g.*, a capture surface) of a solid substrate can be coated with a capture agent (*e.g.*, affinity agent) for an entity (*e.g.*, biological entity) of interest.

[0561] In some embodiments, a set of detection probes provided in a kit may be selected for diagnosis of ovarian cancer.

[0562] In some embodiments, a set of detection probes provided in a kit may be selected for diagnosis of high-grade serous ovarian cancer.

[0563] In some embodiments, a kit may comprise a plurality of sets of detection probes, wherein each set of detection probes is directed for detection of a specific cancer and comprises at least 2 or more detection probes. For example, such a kit can be used to screen a subject for various cancers, one of which is ovarian cancer while other cancers may be selected from skin cancer, lung cancer, breast cancer, colorectal cancer, pancreatic cancer, prostate cancer, brain cancer, and liver cancer) in a single assay.

[0564] In some embodiments, kits provided herein may include instructions for practicing methods described herein. For example, in some embodiments, kits provided herein may include instructions for various aspects of performing an assay for ovarian cancer detection, including, *e.g.*, but not limited to how data may be analyzed and interpreted. These instructions may be present in kits in a variety of forms, one or more of which may be present in the kits. One form in which these instructions may be present is as printed information on a suitable medium or substrate, *e.g.*, a piece or pieces of paper on which the information is printed, in the packaging of kits, in a package insert, *etc.* Yet another means may be a computer readable medium, *e.g.*, diskette, CD, USB drive, *etc.*, on which instructional information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access instructional information. Any convenient means may be present in the kits.

[0565] In some embodiments where kits are for use as companion diagnostics, such kits can include instructions for identifying patients that are likely to respond to a therapeutic agent (*e.g.*, identification of biomarkers that are indicative of patient responsiveness to the therapeutic agent). In some embodiments, such kits can comprise a therapeutic agent for use in tandem with the companion diagnostic test.

[0566] Exemplary embodiments below are also within the scope of the present disclosure:

1. A method comprising steps of:

- (a) providing or obtaining a blood-derived sample from a subject;
- (b) detecting, in the blood-derived sample, extracellular vesicles expressing a first target biomarker signature (“first target biomarker signature-expressing extracellular vesicles”), the first target biomarker signature comprising:

at least one extracellular vesicle-associated surface biomarker and

at least one target biomarker selected from surface biomarkers, wherein:

the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows:

BCAM, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof;

- (c) comparing sample information indicative of level of the first target biomarker signature-expressing extracellular vesicles in the blood-derived sample to reference information including a first reference threshold level;
 - (d) classifying the subject as having or being susceptible to ovarian cancer when the blood-derived sample shows an elevated level of first target biomarker signature-expressing extracellular vesicles relative to a classification cutoff referencing the first reference threshold level.
2. The method of embodiment 1, wherein when the surface biomarker is a polypeptide encoded by the human gene *MUC16*, the polypeptide an intact MUC16 polypeptide.
 3. The method of embodiment 1, wherein when the surface biomarker is a polypeptide encoded by the human gene *MUC16*, the polypeptide a cleaved MUC16 polypeptide.
 4. The method of any one of embodiments 1-3, wherein the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.
 5. The method of any one of embodiments 1-4, wherein when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different.
 6. The method of any one of embodiments 1-5, wherein the steps of (b) and (c) are repeated for at least a second target biomarker signature, and wherein the classification cutoff references the first reference threshold level and at least a second reference threshold level corresponding to the at least a second target biomarker signature.
 7. The method of any one of embodiments 1-6, wherein the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.
 8. The method of any one of embodiments 1-7, wherein the first and/or second target biomarker signature comprises at least one extracellular vesicle-associated surface biomarker and at least two biomarkers selected from the group consisting of: surface biomarkers, intravesicular biomarkers, and intravesicular RNA biomarkers.
 9. The method of any one of embodiments 1-8, wherein the at least two biomarkers comprise one of the following combinations:
 - at least two distinct surface biomarkers;
 - at least two distinct intravesicular biomarkers;
 - at least two distinct intravesicular RNA biomarkers;
 - a surface biomarker and an intravesicular biomarker;
 - a surface biomarker and an intravesicular RNA biomarker; and
 - an intravesicular biomarker and an intravesicular RNA biomarker.

10. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.
11. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.
12. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.
13. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.
14. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
15. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
16. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one

or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof.

17. The method of embodiment 16, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide.
18. The method of embodiment 16, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.
19. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.
20. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.
21. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker *BST2*.
22. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least two target biomarkers, which are *BST2* and *FOLR1*.
23. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least one target biomarker sTn antigen.
24. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *BST2* and *MUC1*.
25. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *FOLR1* and *MUC1*.
26. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *MUC16* and *MSLN*.

27. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are FOLR1 and MUC16.
28. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.
29. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.
30. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.
31. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.
32. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.
33. The method of any one of embodiments 1-9, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.
34. The method of any one of embodiments 1-33, wherein the first or second reference threshold level is determined by levels of target biomarker signature-expressing extracellular vesicles observed in comparable samples from a population of non-cancer subjects.
35. The method of embodiment 34, wherein the population of non-cancer subjects comprises one or more of the following subject populations: healthy subjects, subjects diagnosed with benign tumors, and subjects with non-ovarian-related diseases, disorders, and/or conditions.
36. The method of any one of embodiments 1-35, wherein the blood-derived sample has been subjected to purification (*e.g.*, size exclusion chromatography) to isolate (*e.g.*, directly from the blood-derived sample) nanoparticles having a size range of interest that includes extracellular vesicles.

37. The method of any one of embodiments 1-36, wherein the step of detecting comprises a capture assay.
38. The method of embodiment 37, wherein the capture assay involves contacting the blood-derived sample with a capture agent comprising a target-capture moiety that binds to the at least one extracellular vesicle-associated surface biomarker.
39. The method of embodiment 38, wherein the capture agent is or comprises a solid substrate comprising the target-capture moiety conjugated thereto.
40. The method of embodiment 39, wherein the solid substrate comprises a magnetic bead.
41. The method of any one of embodiments 38-40-, wherein the target-capture moiety is or comprises an antibody agent.
42. The method of any one of embodiments 1-41, wherein the step of detecting comprises a detection assay.
43. The method of any one of embodiments 1-42, wherein the step of detecting comprises a capture assay and a detection assay, the capture assay being performed prior to the detection assay.
44. The method of any one of embodiments 42-43, wherein when the first and/or second target biomarker signature comprises at least one intravesicular RNA biomarkers, the detection assay involves reverse transcription qPCR.
45. The method of any one of embodiments 42-44, wherein when the first and/or second target biomarker signature comprises at least one intravesicular biomarker, the target biomarker signature-expressing extracellular vesicles are processed involving fixation and/or permeabilization prior to the detection assay.
46. The method of any one of embodiments 42-45, wherein when the first and/or second target biomarker signature comprises at least one surface biomarker and/or intravesicular biomarker, the detection assay involves an immunoassay (including, *e.g.*, immuno-PCR, and/or proximity ligation assay).
47. The method of embodiment 46, wherein the detection assay involves a proximity ligation assay.
48. The method of embodiment 47, wherein the proximity ligation assay comprises the steps of:
 - (a) contacting the target biomarker signature-expressing extracellular vesicles that express the at least one extracellular vesicle-associated surface biomarker ("extracellular vesicle-associated surface biomarker-expressing extracellular vesicles") with a set of detection probes, each directed to a target biomarker of the target biomarker signature, which set comprises at least two detection probes, so that a combination comprising the extracellular vesicles and the set of detection probes is generated,

wherein the detection probes each comprise:

 - (i) a target binding moiety directed to the target biomarker of 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle,

(b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the extracellular vesicles such that the at least two detection probes can bind to the same extracellular vesicle that express the target biomarker signature to form a double-stranded complex;

(c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template; and

(d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the blood-derived sample of the target biomarker signature-expressing extracellular vesicles; and

(e) optionally repeating steps a through d at least one additional time using an orthogonal target biomarker signature.

49. The method of embodiment 48, wherein the target binding moiety of the at least two detection probes are directed to the same target biomarker.

50. The method of embodiment 49, wherein the oligonucleotide domain of the at least two detection probes are different.

51. The method of any one of embodiments 42-50, wherein the target-capture moiety of the capture assay is or comprises at least one antibody agent directed to the at least one extracellular vesicle-associated surface biomarker.

52. The method of any one of embodiments 1-51, wherein the method is performed to screen for early-stage ovarian cancer, late-stage ovarian cancer, or recurrent ovarian cancer in the subject.

53. The method of any one of embodiments 1-52, wherein the subject is determined to have a normal plasma CA-125 level.

54. The method of any one of embodiments 1-53, wherein the subject has at least one or more of the following characteristics:

(i) an asymptomatic female (*e.g.*, woman) who is susceptible to ovarian cancer (*e.g.*, at an average population risk (*i.e.*, without hereditary risk) or with hereditary risk for ovarian cancer);

(ii) a post-menopausal woman;

(iii) a female (*e.g.*, woman) with a family history of breast and/or ovarian cancer (*e.g.*, a female (*e.g.*, woman) having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer);

- (iv) a female (*e.g.*, woman) determined to have one or more germline mutations in ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11, TP53, BARD, CHEK2, MRE11A, RAD50, RAD51C, RAD51D and combinations thereof;
 - (v) a female (*e.g.*, woman) with breast cancer determined to have germline mutations in BRCA1, BRCA2 and/or PALB2;
 - (vi) an elderly woman *e.g.*, age 65 or above;
 - (vii) a female (*e.g.*, woman) with one or more non-specific symptoms of ovarian cancer, optionally wherein at least one of the non-specific symptoms is similar to one or more symptoms for irritable bowel syndrome; and
 - (viii) a female (*e.g.*, woman) recommended for CA-125/transvaginal ultrasound (TVUS) periodic screening;
 - (ix) a female (*e.g.*, woman) diagnosed with an imaging-confirmed adnexal mass;
 - (x) a female (*e.g.*, woman) at hereditary risk before undergoing a risk-reducing bilateral salpingo-oophorectomy;
 - (xi) a female (*e.g.*, woman) with a benign gynecological tumor;
 - (xii) a female (*e.g.*, woman) who has been previously treated for ovarian cancer; and
 - (xiii) a female (*e.g.*, woman) with life-history associated risk for ovarian cancer.
55. The method of any one of embodiments 1-54, wherein the method is used in combination with one or more of the following diagnostic assays:
- (i) the subject's annual physical examination (*e.g.*, including a HPV, and/or Pap smear screening for cervical cancer and a mammogram screening for breast cancer).
 - (ii) plasma CA-125 and/or TVUS 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) BRCA1 and/or BRCA2 germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, OVA1 and OVERA tests, and/or circulating tumor cells.
56. The method of any one of embodiments 1-55, wherein the ovarian cancer is high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer.
57. The method of any one of embodiments 1-56, wherein the ovarian cancer is high-grade serous ovarian cancer.
58. The method of embodiment 57, the high-grade serous ovarian cancer is at an early stage.

59. The method of any one of embodiments 1-58, wherein the method is performed to monitor an ovarian cancer patient for response to treatment of an anti-ovarian cancer therapy (*e.g.*, olaparib, cisplatin, rucaparib, niraparib, talazoparib) and/or for cancer recurrence/metastasis.
60. The method of any one of embodiments 1-59 for 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.
61. The method of any one of embodiments 1-59 for detecting cancer, the 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%.
62. The method of any one of embodiments 1-59, comprising steps of: capturing exosomes from a biological sample with a capture agent that selectively interacts with a cancer-specific surface 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 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 biomarkers.
63. The method of any one of embodiments 1-59, 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.
64. The method of any one of embodiments 1-59, 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.
65. The method of any one of embodiments 1-59, 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.
66. The method of any one of embodiments 1-59, comprising detecting marker proximity on exosome surfaces, including an improvement that comprises contacting the exosomes with at

least a pair of binding agents that each comprise a binding moiety and a proximity moiety, wherein the binding moieties are the same and the proximity moieties complement one another; and detecting an interaction between the proximity moieties.

67. A kit for detection of ovarian cancer comprising:

(a) a capture agent comprising a target-capture moiety directed to an extracellular vesicle-associated surface biomarker; and

(b) at least one set of detection probes, which set comprises at least two detection probes each directed to a target biomarker of a target biomarker signature for ovarian cancer, wherein the detection probes each comprise:

(i) a target binding moiety directed at the target biomarker of the target biomarker signature for ovarian cancer; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle;

wherein the target biomarker signature for ovarian cancer comprises:

at least one extracellular vesicle-associated surface biomarker and

at least one target biomarker selected from surface biomarkers, wherein:

the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

68. The kit of embodiment 67, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact *MUC16* polypeptide.

69. The kit of embodiment 67, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved *MUC16* polypeptide.

70. The kit of any one of embodiments 67-69, wherein the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.

71. The kit of any one of embodiments 67-70, wherein when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different.

72. The kit of any one of embodiments 67-71, wherein the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*,

MUC16, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

73. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.
74. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.
75. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.
76. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.
77. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
78. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.

79. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof.
80. The kit of embodiment 79, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact *MUC16* polypeptide.
81. The kit of embodiment 79, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved *MUC16* polypeptide.
82. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T, TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.
83. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.
84. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a *MUC1* polypeptide, and (ii) at least one target biomarker *BST2*.
85. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a *MUC1* polypeptide, and (ii) at least two target biomarkers, which are *BST2* and *FOLR1*.
86. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a *MUC16* polypeptide, and (ii) at least one target biomarker sTn antigen.
87. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *BST2* and *MUC1*.
88. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *FOLR1* and *MUC1*.
89. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is

or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and MSLN.

90. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are FOLR1 and MUC16.
91. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.
92. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.
93. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.
94. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.
95. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.
96. The kit of any one of embodiments 67-72, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.
97. The kit of any one of embodiments 67-96, wherein the target binding moiety of the at least two detection probes is each directed to the same target biomarker of the target biomarker signature.
98. The kit of any one of embodiments 67-96, wherein the oligonucleotide domain of the at least two detection probes are different.
99. The kit of any one of embodiments 67-96, wherein the target binding moiety of the at least two detection probes is each directed to a distinct target biomarker of the target biomarker signature.
100. The kit of any one of embodiments 67-99, further comprising at least one additional reagent (*e.g.*, a ligase, a fixation agent, and/or a permeabilization agent).

101. The kit of any one of embodiments 67-100, comprising at least two sets (including, *e.g.*, at least three sets) of detection probes, which each set comprises at least two detection probes each directed to a target biomarker of a distinct target biomarker signature for ovarian cancer.
102. The kit of any one of embodiments 67-96, comprising:
- (a) a first capture agent comprising a target-capture moiety;
 - (b) a second capture agent comprising a target-capture moiety;
 - (c) at least two sets of detection probes, wherein the detection probes each comprise:
 - (i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle.
103. The kit of any one of embodiments 67-96, comprising:
- (a) a first capture agent comprising a target-capture moiety;
 - (b) a second capture agent comprising a target-capture moiety;
 - (c) a third capture agent comprising a target-capture moiety;
 - (d) at least three sets of detection probes, wherein the detection probes each comprise:
 - (i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same extracellular vesicle.
104. A complex comprising:
- (a) an extracellular vesicle expressing a target biomarker signature for ovarian cancer, wherein the target biomarker signature comprises:
 - at least one extracellular vesicle-associated surface biomarker and
 - at least one target biomarker selected from surface biomarkers, wherein:
 the surface biomarkers are selected from (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof;

wherein the extracellular vesicle is immobilized onto a solid substrate comprising a target-capture moiety directed to the extracellular vesicle-associated surface biomarker;

(b) a first detection probe and a second detection probe each bound to the extracellular vesicle, wherein each detection probe comprises:

(i) a target binding moiety directed to one of the target biomarker of the tumor 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 first and second detection probes are hybridized to each other.

105. The complex of embodiment 104, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide.

106. The complex of embodiment 104, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.

107. The complex of any one of embodiments 104-106, wherein the first target biomarker signature further comprises an intravesicular biomarker and/or an intravesicular RNA biomarker.

108. The complex of any one of embodiments 104-107, wherein when the at least one target biomarker is selected from one or more of the surface biomarkers, the selected surface biomarker(s) and the at least one extracellular vesicle-associated surface biomarker are different;

109. The complex of any one of embodiments 104-108, wherein the extracellular vesicle-associated surface biomarker is or comprises (i) polypeptides encoded by human genes as follows: *BST2*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

110. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more target surface biomarkers, which include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

111. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.

112. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *BST2*; and (ii) one or more target surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.
113. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.
114. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC1*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
115. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a polypeptide encoded by human gene *MUC16*; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
116. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof.
117. The complex of embodiment 116, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide.
118. The complex of embodiment 116, wherein the surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide a cleaved MUC16 polypeptide.
119. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a carbohydrate-dependent marker comprising Thomsen-Friedenreich (T,

- TF) antigen; and (ii) one or more target surface biomarkers, which include at least one polypeptide encoded by human gene *BST2*.
120. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker *BST2*.
121. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker *BST2*.
122. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least two target biomarkers, which are *BST2* and *FOLR1*.
123. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least one target biomarker sTn antigen.
124. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *BST2* and MUC1.
125. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are *FOLR1* and MUC1.
126. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and MSLN.
127. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are *FOLR1* and MUC16.
128. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC16 polypeptide, and (ii) at least two target biomarkers, which are MUC1 and MUC16.

129. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least one target biomarker SLC34A2.
130. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a T antigen, and (ii) at least one target biomarker BST2.
131. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a sTn antigen, and (ii) at least two target biomarkers, which are MUC16 and cleaved MUC16.
132. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a CA19-9 antigen, and (ii) at least two target biomarkers, which are BST2 and MUC16.
133. The complex of any one of embodiments 104-109, wherein the first and/or second target biomarker signature comprises (i) at least one extracellular vesicle-associated surface biomarker, which is or comprises a MUC1 polypeptide, and (ii) at least one target biomarker BCAM.
134. The complex of any one of embodiments 104-133, wherein the target binding moiety of the at least two detection probes is each directed to the same target biomarker of the target biomarker signature.
135. The complex of embodiment 134, wherein the oligonucleotide domain of the at least two detection probes are different.
136. The complex of any one of embodiments 104-133, wherein the target binding moiety of the at least two detection probes is each directed to a distinct target biomarker of the target biomarker signature.
137. The complex of any one of embodiments 104-136, wherein the solid substrate comprises a magnetic bead.
138. The complex of any one of embodiments 104-137, wherein the target-capture moiety is or comprises an antibody agent.
139. The complex of any one of embodiments 104-138, 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.

140. The complex of any one of embodiments 104-138, 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.
141. The complex of any one of embodiments 104-138, 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.
142. A set of probes for use in a method, kit, or complex of any one of embodiments 1-141, wherein each set of probes 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.
143. A method comprising steps of:
 - (a) providing or obtaining a blood-derived sample from a female subject;
 - (b) detecting, in the blood-derived sample, extracellular vesicles expressing a first target biomarker signature ("first target biomarker signature-expressing extracellular vesicles"), the first target biomarker signature comprising:
 - at least one extracellular vesicle-associated surface biomarker; and
 - at least one target surface biomarker,wherein the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker are each independently selected from:
 - (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; and
 - (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (iii) combinations thereof;

- (c) comparing sample information indicative of level of the first target biomarker signature-expressing extracellular vesicles in the blood-derived sample to reference information including a first reference threshold level;
 - (d) classifying the subject as having or being susceptible to ovarian cancer when the blood-derived sample shows an elevated level of the first target biomarker signature-expressing extracellular vesicles relative to a classification cutoff referencing the first reference threshold level.
144. The method of embodiment 143, wherein the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker are different.
145. The method of any one of embodiments 143-144, wherein the steps of (b) and (c) are repeated for at least a second target biomarker signature, and wherein the classification cutoff references the first reference threshold level and at least a second reference threshold level corresponding to the at least a second target biomarker signature.
146. The method of any one of embodiments 143-144, wherein the steps of (b) and (c) are repeated for a plurality of additional target biomarker signatures, and wherein the classification cutoff references each reference threshold level corresponding to each target biomarker signature.
147. The method of any one of embodiments 143-146, wherein the first target biomarker signature or at least one of the target biomarker signatures comprises at least one extracellular vesicle-associated surface biomarker and at least one target surface biomarker, which combination is selected from the following:
- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
 - (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*; and
 - (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
148. The method of any one of embodiments 143-146, wherein the first target biomarker signature or at least one of the target biomarker signatures comprises at least one extracellular vesicle-associated surface biomarker and at least two target surface biomarkers, which combination is selected from the following:
- (i) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
 - (ii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
 - (iii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and

- (iv) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.
149. The method of any one of embodiments 146-148, wherein the first target biomarker signature and the plurality of additional target biomarker signatures collectively comprise the following combinations of the at least one extracellular vesicle-associated surface biomarker and the at least one target surface biomarker:
- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
 - (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
 - (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
 - (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
 - (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
 - (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
 - (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.
150. The method of any one of embodiments 143-149, wherein the reference threshold level(s) is/are determined by level(s) of the corresponding target biomarker signature-expressing extracellular vesicles observed in comparable samples from a population of non-cancer subjects.
151. The method of embodiment 150, wherein the population of non-cancer subjects comprises one or more of the following subject populations: healthy subjects, subjects diagnosed with benign tumors, and subjects with non-ovarian-related diseases, disorders, and/or conditions.
152. The method of any one of embodiments 143-151, wherein the blood-derived sample has been subjected to purification (*e.g.*, size exclusion chromatography) to isolate (*e.g.*, directly from the blood-derived sample) nanoparticles having a size range of interest that includes extracellular vesicles.
153. The method of any one of embodiments 143-152, wherein the step of detecting comprises a capture assay.
154. The method of embodiment 153, wherein the capture assay involves contacting the blood-derived sample with a capture probe comprising a target-capture moiety that binds to the at least one extracellular vesicle-associated surface biomarker.

155. The method of embodiment 154, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to the at least one extracellular vesicle-associated surface biomarker.
156. The method of embodiment 154 or 155, wherein the at least one extracellular vesicle-associated surface biomarker is or comprises a sialyl Lewis A antigen (also known as CA19-9), a polypeptide encoded by human gene MUC1, a polypeptide encoded by human gene MUC16, or a sialyl Tn (sTn) antigen.
157. The method of any one of embodiments 154-156, wherein the capture probe is or comprises a solid substrate comprising the target-capture moiety conjugated thereto.
158. The method of embodiment 157, wherein the solid substrate comprises a magnetic bead.
159. The method of any one of embodiments 143-158, wherein the step of detecting comprises a detection assay.
160. The method of any one of embodiments 143-159, wherein the step of detecting comprises a capture assay and a detection assay, the capture assay being performed prior to the detection assay.
161. The method of embodiment 159 or 160, wherein the detection assay involves an immunoassay (including, *e.g.*, immuno-PCR, and/or proximity ligation assay).
162. The method of any one of embodiments 159-161, wherein the detection assay involves a proximity ligation assay.
163. The method of embodiment 162, wherein the proximity ligation assay comprises the steps of:
 - (a) contacting extracellular vesicles in the blood-derived sample with a set of detection probes, each directed to the at least one target surface biomarker of the target biomarker signature, which set comprises at least two detection probes, so that a complex comprising the extracellular vesicles and the set of detection probes is generated,

wherein the detection probes each comprise:

 - a target binding moiety directed to one of the at least one target surface biomarker of the target biomarker signature; and
 - 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle,
 - (b) maintaining the combination under conditions that permit binding of the set of detection probes to their respective targets on the extracellular vesicles such that the at least two detection probes

can bind to the same extracellular vesicle that express the target biomarker signature to form a double-stranded complex;

- (c) contacting the double-stranded complex with a nucleic acid ligase to generate a ligated template;
- (d) detecting the ligated template, wherein presence of the ligated template is indicative of presence in the blood-derived sample of the target biomarker signature-expressing extracellular vesicles; and
- (e) optionally repeating steps (a) through (d) at least one additional time using an orthogonal target biomarker signature.

- 164. The method of embodiment 163, wherein the target binding moieties of the at least two detection probes are each directed to the same target surface biomarker.
- 165. The method of embodiment 164, wherein the oligonucleotide domain of the at least two detection probes are different.
- 166. The method of embodiment 164 or 165, wherein the same target surface biomarker is or comprises a polypeptide encoded by human gene *BST2*.
- 167. The method of embodiment 166, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*.
- 168. The method of embodiment 164 or 165, wherein the same target surface biomarker is or comprises a sialyl Tn (sTn) antigen.
- 169. The method of embodiment 168, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.
- 170. The method of embodiment 163, wherein the target binding moieties of the at least two detection probes are each directed to a distinct target surface biomarker.
- 171. The method of embodiment 170, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*.
- 172. The method of embodiment 171, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*.
- 173. The method of embodiment 170, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
- 174. The method of embodiment 170, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.

175. The method of embodiment 170, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*.
176. The method of any one of embodiments 173-175, wherein the target-capture moiety of the capture assay is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.
177. The method of any one of embodiments 143-176, wherein the method is performed to screen for early-stage ovarian cancer, late-stage ovarian cancer, or recurrent ovarian cancer in the subject.
178. The method of any one of embodiments 143-176, wherein the method is performed to screen for early-stage ovarian cancer.
179. The method of any one of embodiments 143-178, wherein the subject has at least one or more of the following characteristics:
- (i) an asymptomatic female (*e.g.*, woman) who is susceptible to ovarian cancer (*e.g.*, at an average population risk (*i.e.*, without hereditary risk) or with hereditary risk for ovarian cancer);
 - (ii) a post-menopausal woman;
 - (iii) a female (*e.g.*, woman) with a family history of breast and/or ovarian cancer (*e.g.*, a female (*e.g.*, woman) having one or more first-degree relatives with a history of breast cancer and/or ovarian cancer);
 - (iv) a female (*e.g.*, woman) determined to have one or more germline mutations in ATM, BRCA1, BRCA2, CDKN2A, MSH2, MLH1, MSH2, EPCAM, PALB2, STK11, TP53, BARD, CHEK2, MRE11A, RAD50, RAD51C, RAD51D and combinations thereof;
 - (v) a female (*e.g.*, woman) with breast cancer determined to have germline mutations in BRCA1, BRCA2 and/or PALB2;
 - (vi) an elderly woman *e.g.*, age 65 or above;
 - (vii) a female (*e.g.*, woman) with one or more non-specific symptoms of ovarian cancer, optionally wherein at least one of the non-specific symptoms is similar to one or more symptoms for irritable bowel syndrome; and
 - (viii) a female (*e.g.*, woman) recommended for plasma CA-125/transvaginal ultrasound (TVUS) periodic screening;
 - (ix) a female (*e.g.*, woman) diagnosed with an imaging-confirmed adnexal mass;
 - (x) a female (*e.g.*, woman) at hereditary risk for ovarian cancer before undergoing a risk-reducing bilateral salpingo-oophorectomy;
 - (xi) a female (*e.g.*, woman) with a benign gynecological tumor;
 - (xii) a female (*e.g.*, woman) who has been previously treated for ovarian cancer; and
 - (xiii) a female (*e.g.*, woman) with life-history associated risk for ovarian cancer.

180. The method of any one of embodiments 143-179, wherein the subject is determined to have a normal serum CA-125 level (*e.g.*, equal to or lower than 25 U/mL).
181. The method of any one of embodiments 143-178, wherein the female subject is diagnosed with an imaging-confirmed adnexal mass.
182. The method of embodiment 181, wherein the female subject is determined to have an elevated plasma or serum CA-125 level (*e.g.*, greater than 25 U/mL).
183. The method of any one of embodiments 143-182, wherein the method is used in combination with one or more of the following diagnostic assays:
 - (i) the subject's annual physical examination (*e.g.*, including a HPV, and/or Pap smear screening for cervical cancer and a mammogram screening for breast cancer).
 - (ii) plasma or serum CA-125 and/or TVUS 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) BRCA1 and/or BRCA2 germline and somatic mutation assays, or assays involving cell-free tumor DNA, liquid biopsy, serum protein and cell-free DNA, OVA1 and OVERA tests, and/or circulating tumor cells.
184. The method of any one of embodiments 143-183, wherein the ovarian cancer is high-grade serous ovarian cancer, endometrioid ovarian cancer, clear-cell ovarian cancer, low-grade serous ovarian cancer, or mucinous ovarian cancer.
185. The method of any one of embodiments 143-183, wherein the ovarian cancer is high-grade serous ovarian cancer.
186. The method of embodiment 185, the high-grade serous ovarian cancer is at an early stage.
187. A method for differentiating benign adnexal mass from ovarian cancer, wherein the method comprises:
 - (a) detecting, in a blood-derived sample from a female subject determined to have an adnexal mass, on surfaces of intact extracellular vesicles co-localization of at least one biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:
 - (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; and
 - (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (iii) combinations thereof;
 - (b) comparing the detected co-localization level with a reference level; and

- (c) identifying the adnexal mass of the female subject to be likely benign when the detected co-localization level is or comparable to the reference level; or identifying the adnexal mass to be cancerous when the detected co-localization level is above the reference level.
188. The method of embodiment 187, wherein the method for differentiating benign adnexal mass from ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95%.
189. The method of embodiment 187 or 188, wherein the female subject is determined to have an elevated serum CA-125 level (*e.g.*, greater than 25 U/mL).
190. A method for detection of early-stage ovarian cancer, wherein the method comprises:
- (a) detecting, in a blood-derived sample from a female subject, on surfaces of intact extracellular vesicles co-localization of at least one biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:
 - (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*;
 - (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (iii) combinations thereof;
 - (b) comparing the detected co-localization level with a reference level; and
 - (c) identifying the female subject to be negative for ovarian cancer when the detected co-localization level is or comparable to the reference level; or identifying the female subject as likely to have or be susceptible to ovarian cancer, when the detected co-localization level is above the reference level.
191. The method of embodiment 190, wherein the method for detection of early-stage ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 80% to 95%.
192. The method of embodiment 190 or 191, wherein the female subject is determined to have a normal plasma or serum CA-125 level (*e.g.*, less than or equal to 25 U/mL).
193. The method of any one of embodiments 187-192, wherein the detecting comprises detecting on surfaces of intact extracellular vesicles co-localization of the at least one biomarker combination, wherein the at least one biomarker combination is selected from one of the following:
- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
 - (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
 - (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.

- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

194. The method of any one of embodiments 187-192, wherein the detecting comprises detecting on surfaces of intact extracellular vesicles co-localization of each of the following biomarker combinations:

- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen;
- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

195. The method of any one of embodiments 187-194, wherein the detecting comprises:

- (a) capturing the intact extracellular vesicles from the blood-derived sample with a capture probe that selectively interacts with the at least one capture biomarker on the intact extracellular vesicles;
- (b) contacting the captured extracellular vesicles with at least one set of at least two detection probes that each selectively interacts with the at least one detection biomarker on the intact extracellular vesicles; and
- (c) detecting a product formed when the at least two detection probes of the set are in sufficiently close proximity on the individual extracellular vesicles.

196. The method of embodiment 195, wherein the capture probe comprises a target-capture moiety that binds to the capture biomarker.

197. The method of embodiment 196, wherein the target-capture moiety is or comprises an antibody agent directed to the capture biomarker.

198. The method of any one of embodiments 187-197, wherein the capture biomarker is or comprises a sialyl Lewis A antigen (also known as CA19-9), a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *MUC16*, or a sialyl Tn (sTn) antigen.
199. The method of any one of embodiments 195-198, wherein the capture probe is or comprises a solid substrate comprising the target-capture moiety conjugated thereto.
200. The method of embodiment 199, wherein the solid substrate comprises a magnetic bead.
201. The method of any one of embodiments 195-200, wherein the at least two detection probes each comprise:
- (i) a target binding moiety directed to one of the at least detection biomarker; 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 characterized in that they can hybridize to each other when the detection probes are bound to the same extracellular vesicle.
202. The method of any one of embodiments 195-201, wherein the product was formed when the at least two detection probes of the set are in sufficiently close proximity on the individual extracellular vesicles such that the single-stranded overhang portions of the at least two detection probes of the set hybridize to each other to form a double-stranded complex.
203. The method of embodiment 202, wherein the product formed comprises a ligated template upon contacting the double-stranded complex with a nucleic acid ligase.
204. The method of any one of embodiments 195-203, wherein the target binding moieties of the at least two detection probes are each directed to the same detection biomarker.
205. The method of embodiment 204, wherein the oligonucleotide domain of the at least two detection probes are different.
206. The method of embodiment 204 or 205, wherein the same detection biomarker is or comprises a polypeptide encoded by human gene *BST2*.
207. The method of embodiment 206, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*.
208. The method of embodiment 204 or 205, wherein the same detection biomarker is or comprises a sialyl Tn (sTn) antigen.
209. The method of embodiment 208, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.
210. The method of embodiment 195-203, wherein the target binding moieties of the at least two detection probes are each directed to a distinct detection biomarker.

211. The method of embodiment 210, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*.
212. The method of embodiment 211, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*.
213. The method of embodiment 210, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
214. The method of embodiment 210, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
215. The method of embodiment 210, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*.
216. The method of any one of embodiments 213-215, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.
217. A kit comprising:
 at least one set of probes for a biomarker combination specific for detection of ovarian cancer,
 wherein the biomarker combination comprises at least one capture biomarker on exosomes and at least one detection biomarker on exosomes, and wherein the capture biomarker and the detection biomarker are each independently selected from:
 (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*;
 (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 (iii) combinations thereof; and
 wherein the at least one set of probes comprises:
 a capture probe comprising a target-capture moiety directed to the capture biomarker; and
 at least two detection probes each comprising a target binding moiety directed to the at least one detection biomarker.
218. The kit of embodiment 217, comprising a plurality of sets of probes, each set for a distinct biomarker combination specific for detection of ovarian cancer.
219. The kit of embodiment 217 or 218, wherein the biomarker combination(s) is/are selected from one of the following:

- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

220. The kit of embodiment 217 or 218, comprising at least 7 sets of probes, each set for a distinct biomarker combination as follows:

- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

221. The kit of any one of embodiments 217-220, wherein the capture probe and detection probes selectively bind to respective biomarkers on the exosomes with a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95%.

222. The kit of any one of embodiments 217-221, wherein the detection probes each further comprises 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same exosome.

223. The kit of any one of embodiments 217-222, wherein the target binding moieties of the at least two detection probes are each directed to the same detection biomarker on the exosomes.
224. The kit of embodiment 223, wherein the oligonucleotide domain of the at least two detection probes are different.
225. The kit of embodiment 223 or 224, wherein the same detection biomarker is or comprises a polypeptide encoded by human gene *BST2*.
226. The kit of embodiment 225, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*.
227. The kit of embodiment 223 or 224, wherein the same detection biomarker is or comprises a sialyl Tn (sTn) antigen.
228. The kit of embodiment 227, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.
229. The kit of embodiment 217-222, wherein the target binding moieties of the at least two detection probes are each directed to a distinct detection biomarker on the exosomes.
230. The kit of embodiment 229, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*.
231. The kit of embodiment 230, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*.
232. The kit of embodiment 229, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
233. The kit of embodiment 229, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
234. The kit of embodiment 229, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*.
235. The kit of any one of embodiments 232-234, wherein the target-capture moiety of the capture probe is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.
236. The kit of any one of embodiments 217-235, further comprising at least one additional reagent (*e.g.*, a ligase, a fixation agent, and/or a permeabilization agent).

[0567] Other features of the invention will become apparent in the course of the following description of exemplary embodiments, which are given for illustration of the invention and are not intended to be limiting thereof.

EXEMPLIFICATION

Example 1: Detection of an exemplary target biomarker signature in individual extracellular vesicles associated with ovarian cancer

[0568] The present Example describes synthesis of detection probes for targets (*e.g.*, target biomarker(s)) each comprising a target-binding moiety and an oligonucleotide domain (comprising a double-stranded portion and a single stranded overhang) coupled to the target-binding moiety. The present Example further demonstrates that use of such detection probes to detect the presence or absence of biological entities (*e.g.*, extracellular vesicles) comprising two or more distinct targets.

[0569] In some embodiments, a detection probe can comprise a double-stranded oligonucleotide with an antibody agent specific to a target cancer biomarker at one end and a single stranded overhang at another end. When two or more detection probes are bound to the same biological entity (*e.g.*, an extracellular vesicle), the single-stranded overhangs of the detection probes are in close proximity such that they can hybridize to each other to form a double-stranded complex, which can be subsequently ligated and amplified for detection.

[0570] This study employed at least two detection probes in a set. In some embodiments, such at least two detection probes are directed to the same target biomarker. In some embodiments, such at least two detection probes directed to the same target, which may be directed to different epitopes of the same target or to the same epitope of the same target. In some embodiments, such at least two detection probes are directed to distinct targets. A skilled artisan reading the present disclosure will understand that two detection probes can be directed to different target biomarkers, or that three or more detection probes, each directed towards a distinct target protein, may be used. Further, compositions and methods described in this Example can be extended to applications in different biological samples (*e.g.*, comprising extracellular vesicles).

[0571] The present Example shows experimental data from certain experiments demonstrating technologies provided herein are capable of detecting ovarian cancer (*e.g.*, HGSOC) in patient samples using exemplary biomarker combinations as described herein

Overview of an exemplary assay

[0572] In some embodiments, a target entity detection system described herein is a duplex system. In some embodiments, such a duplex system, *e.g.*, as illustrated in **Fig. 2**, utilizes two antibodies that each recognize a different epitope. Paired double-stranded template DNAs are also utilized in qPCR, each of which has specific four-base 5' overhangs complementary to the 5' overhang on its partner. Each antibody is conjugated with one of the two double-stranded DNA templates. When the antibodies bind their target epitopes, the sticky ends of the respective templates can hybridize. These sticky ends are then ligated together, for example, by T7 ligase, prior to PCR

amplification. For hybridization between the two DNA templates to occur, the two antibodies need to be bound close enough to each other (within 50 to 60 nm, the length of the DNA linker and antibody). Any templates that bind but remain unligated will not produce PCR product, as shown in *Fig. 2*.

Exemplary Methods:

Oligonucleotides

[0573] In some embodiments, oligonucleotides can have the following sequence structure and modifications. It is noted that the strand numbers below correspond to the numerical values associated with strands shown in *Fig. 2*.

Strand 1 v1:

/5AzideN/CAGTCTGACACAGCAGTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGCTA GACAGAGGTGT, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/CAGTCTGACACAGCAGTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGCTA GACAGAGGTGT, where /5AmMC12/ refers to an amine group (*e.g.*, a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/CAGTCTGACACAGCAGTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGC TAGACAGAGGTGT, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer.

Strand 2 v1:

/5AzideN/GACCTGACCTACAGTGACCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTCCT GGTCTCACTAG, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/GACCTGACCTACAGTGACCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTCCT GGTCTCACTAG, where /5AmMC12/ refers to an amine group (*e.g.*, a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/GACCTGACCTACAGTGACCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTC CTGGTCTCACTAG, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer

Strand 3 v1:

/5Phos/GAGTACACCTCTGTCTAGCCAGTCACGGATGTCAAGGGTAGCAGCGACGATTAACGACTG CTGTGTCAGACTG, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 4 v1:

/5Phos/ACTCCTAGTGAGACCAGGAGCCAACTGGACAGTGGCTAATCAGGCAAGGCTATGGTCAC

TGTAGGTCAGGTC, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 5 v1:

CAGTCTGACACAGCAGTCGT

Strand 6 v1:

GACCTGACCTACAGTGACCA

Strand 7 (Probe) v1:

/56-FAM/TGGCTAGAC/ZEN/AGAGGTGTACTCCTAGTGAGA/3IABkFQ/, wherein /56-FAM/ refers to a fluorescein (e.g., 6-FAM) at the 5' oligonucleotide terminus; and /3IABkFQ/ refers to a fluorescein quencher at the 3' oligonucleotide terminus.

[0574] In some embodiments, oligonucleotides can have the following sequence structure and modifications. It is noted that the strand numbers below correspond to the numerical values associated with strands shown in *Fig. 2*.

Strand 1 v2:

/5AzideN/CAGTCTGACTCACCACCTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGCTAGACAGAGGTGT, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/CAGTCTGACTCACCACCTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGCTAGACAGAGGTGT, where /5AmMC12/ refers to an amine group (e.g., a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/CAGTCTGACTCACCACCTCGTTAATCGTCGCTGCTACCCTTGACATCCGTGACTGGCTAGACAGAGGTGT, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer

Strand 2 v2:

/5AzideN/CACCAGACCTACGAAGTCCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTCCTGGTCTCACTAG, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/CACCAGACCTACGAAGTCCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTCCTGGTCTCACTAG, where /5AmMC12/ refers to an amine group (e.g., a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/CACCAGACCTACGAAGTCCATAGCCTTGCCTGATTAGCCACTGTCCAGTTTGGCTCCTGGTCTCACTAG, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer

Strand 3 v2:

/5Phos/GAGTACACCTCTGTCTAGCCAGTCACGGATGTCAAGGGTAGCAGCGACGATTAAACGAGTG

GTGAGTCAGACTG, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 4 v2:

/5Phos/ACTCCTAGTGAGACCAGGAGCCAACTGGACAGTGGCTAATCAGGCAAGGCTATGGACTTCGTAGGTCTGGTG, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 5 v2:

CAGTCTGACTCACCCTCGT

Strand 6 v2:

CACCAGACCTACGAAGTCCA

Strand 7 (Probe) v2:

/56-FAM/TGGCTAGAC/ZEN/AGAGGTGTACTCCTAGTGAGA/3IABkFQ/, wherein /56-FAM/ refers to a fluorescein (e.g., 6-FAM) at the 5' oligonucleotide terminus; and /3IABkFQ/ refers to a fluorescein quencher at the 3' oligonucleotide terminus.

[0575] In some embodiments, oligonucleotides can have the following sequence structure and modifications. It is noted that the strand numbers below correspond to the numerical values associated with strands shown in *Fig. 2*.

Strand 1 v1-med:

/5AzideN/CAGTCTGACACAGCAGTCGTGACTGGCTAGACAGAGGTGT, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/CAGTCTGACACAGCAGTCGTGACTGGCTAGACAGAGGTGT, where /5AmMC12/ refers to an amine group (e.g., a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/CAGTCTGACACAGCAGTCGTGACTGGCTAGACAGAGGTGT, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer.

Strand 2 v1-med:

/5AzideN/GACCTGACCTACAGTGACCATTGGCTCCTGGTCTCACTAG, where /5AzideN/ refers to an azide group linked to the 5' oligonucleotide terminus via a NHS ester linker, or

/5AmMC12/GACCTGACCTACAGTGACCATTGGCTCCTGGTCTCACTAG, where /5AmMC12/ refers to an amine group (e.g., a primary amino group) linked to the 5' oligonucleotide terminus via a 12-carbon spacer, or

/5ThiolMC6/GACCTGACCTACAGTGACCATTGGCTCCTGGTCTCACTAG, where /5ThiolMC6/ refers to a thiol linked to the 5' oligonucleotide terminus via a 6-carbon spacer

Strand 3 v1-med:

/5Phos/GAGTACACCTCTGTCTAGCCAGTCACGACTGCTGTGTCAGACTG, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 4 v1-med:

/5Phos/ACTCCTAGTGAGACCAGGAGCCAATGGTCACTGTAGGTCAGGTC, wherein /5Phos/ refers to a phosphate group linked to the 5' oligonucleotide terminus

Strand 5 v1:

CAGTCTGACACAGCAGTCGT

Strand 6 v1:

GACCTGACCTACAGTGACCA

Strand 7 (Probe) v1:

/56-FAM/TGGCTAGAC/ZEN/AGAGGTGTACTCCTAGTGAGA/3IABkFQ/, wherein /56-FAM/ refers to a fluorescein (e.g., 6-FAM) at the 5' oligonucleotide terminus; and /3IABkFQ/ refers to a fluorescein quencher at the 3' oligonucleotide terminus.

Antibody-oligonucleotide (e.g., antibody-DNA) conjugation:

[0576] Antibody aliquots ranging from 25-100 µg were conjugated with oligonucleotide strands. For example, 60 µg aliquots of antibodies directed to target surface biomarkers were conjugated with hybridized strands 1+3 and 2+4, for example, using copper-free click chemistry. The first step was to prepare DBCO-functionalized antibodies to participate in the conjugation reaction with azide-modified oligonucleotide domain (e.g., DNA domain). This began with reacting the antibodies with the DBCO-PEG5-NHS heterobifunctional cross linker. The reaction between the NHS ester and available lysine groups was allowed to take place at room temperature for 2 hours, after which unreacted crosslinker may be removed using centrifugal ultrafiltration. To complete the conjugation, azide-modified oligonucleotide domains (e.g., DNA domain) and the DBCO-functionalized antibodies was allowed to react overnight at room temperature. The concentration of conjugated antibody was measured, for example, using the Qubit protein assay.

Cell Culture

[0577] Negative control cells (e.g., non-ovarian cancer cells such as melanoma cells or healthy cells or cells from benign adnexal mass) may be grown in Eagle's Minimum Essential Medium (EMEM) with 10% exosome-free FBS and 50 units of penicillin/streptomycin per mL. Ovarian cancer cells may be grown in Roswell Park Memorial Institute (RPMI 1640) with 10% exosome-free FBS and 50 units of penicillin/streptomycin per mL. There are currently dozens, if not more, exemplary ovarian cancer cell lines that may be useful to develop an assay for detection of ovarian cancer. Cell lines may be grown in complete media supplemented with exosome-depleted fetal bovine serum per the recommendation of the cell line supplier or inventor.

Purification of extracellular vesicles from cell culture medium

[0578] In some embodiments, ovarian cancer cells and negative control cells may be grown in their respective media until they reach ~80% confluence. The cell culture medium may be

collected and spun at 300 RCF for 5 minutes at room temperature (RT) to remove cells and debris. The supernatant may then be collected and used in assays as described herein or frozen at -80°C .

Thawing

[0579] If prior to use, samples were stored at -80°C , they are thawed. In brief, 50 mL tubes containing frozen conditioned media placed in plastic racks, the racks are placed in an empty ice bucket. Room temperature (RT) water is added, and samples are allowed to thaw, with periodic inversion/shaking to facilitate thawing. Tubes are consolidated such that all the tubes for each cell line are the same volume. A typical purification volume is approximately 200 mLs of spent medium per cell line. If larger batches are desired, this volume can be increased.

Clarification

[0580] In some embodiments, samples are clarified prior to use. Clarification of media serves to remove cells and debris. In brief, 1) spin at 1300 RCF for 10 mins; transfer supernatant to a new 50 mL conical tube using a pipette, leaving ~ 1 cm of medium (to avoid disturbing the pellet), the remaining media is not decanted; 2) spin at 2000 RCF for 30 mins; transfer supernatant to a new 50 mL conical tube using a pipette, leaving ~ 1 cm of medium (to avoid disturbing the pellet), the remaining media is not decanted.

Concentrate Media

[0581] In some embodiments, samples are concentrated. In brief: 1) a single 15 mL 10 kDa MWCO filter is used for approximately 100 mLs of medium (for example, for a 200 mL batch, two 10 kDa MWCO ultrafiltration tubes will be needed). In some embodiments, the same ultrafiltration column can be sequentially added to and re-spun to enable the concentration of large volumes of medium. In general, columns were utilized according to the manufacturer's protocol. Columns are spun for 10-12 minutes each time, at maximum speed (2500 to 4,300 RCF). 2) When each of the two tubes containing the same spent medium reaches ~ 1500 uL, the two tubes are combined into one, the now empty tube may be utilized as a balance. 3) When removing the concentrated medium, the sides of the concentration chamber may be flushed to release as many entrapped EVs as possible, while avoiding frothing, the consolidated media may be concentrated until there is 1 mL left. 4) The media is transferred to a 1.5 mL protein LoBind tube, with the 1 mL line marked, if necessary, volume is corrected to 1 mL with 20 nm filtered 1x PBS.

Final Clarification Spin

[0582] To remove any remaining debris, the concentrated media can be centrifuged at 10,000 RCF for 10 minutes at 21°C in a tabletop Eppendorf centrifuge.

Run Concentrated Media Through Prepared IZON Columns

[0583] Izon columns are washed as described by the manufacturer, 20 nm filtered 1X PBS can be used to both wash the columns and recover the samples. 1 mL of concentrated spent medium

can be run through the column and fractions can be collected (*e.g.*, fractions 7, 8, and 9) in 5-mL Eppendorf flip-cap tubes, following the manufacturer's protocol.

Particle counts:

[0584] Particle counts was obtained, *e.g.*, using a SpectraDyne particle counting instrument using the TS400 chips, to measure nanoparticle range between 65 and 1000 nm. In some embodiments, a particle size that is smaller than 65 nm or larger than 1000 nm.

Generation of patient plasma pools:

[0585] In some embodiments, pooled patient plasma pools were utilized. In brief, 1 mL aliquots of patient plasma were thawed at room temperature for at least 30 minutes. The tubes were vortexed briefly and spun down to consolidate plasma to the bottom of each tube. Plasma samples from a given patient cohort were combined in an appropriately sized container and mixed thoroughly by end-over-end mixing. Each plasma pool was split into 1 mL aliquots in Protein Lo-bind 1.5 mL Eppendorf tubes and refrozen at -80°C.

Whole-plasma clarification (optional):

[0586] In some embodiments, prior to EVs purification, samples were blinded by personnel who would not participate in sample-handling. The patient-identification information was revealed after the experiment was completed to enable data analysis. 1 mL aliquots of whole plasma were removed from storage at -80°C and subjected to three clarification spins to remove cells, platelets, and debris.

Size-exclusion chromatography purification of EVs from clarified plasma:

[0587] Each clarified plasma sample (individual samples or pooled samples) was run through a single-use, size-exclusion purification column to isolate the EVs. Nanoparticles having a size range of about 65 nm to about 1000 nm were collected for each sample. In some embodiments, smaller particle range may be desirable.

Capture-antibody conjugation to magnetic-capture beads:

[0588] Antibodies were conjugated to magnetic beads (*e.g.*, epoxy-functionalized Dynabeads™). Briefly, beads were weighed in a sterile environment and resuspended in buffer. Antibodies were, for example, at approximately 8 µg of Ab per mg of bead, mixed with the functionalized beads and the conjugation reaction took place overnight at 37°C with end-over-end mixing. The beads were washed several times using the wash buffer provided by the conjugation kit and were stored at 4°C in the provided storage buffer, or at -20°C in a glycerol-based storage buffer.

Direct capture of purified plasma EVs using antibody-conjugated magnetic beads:

[0589] In certain embodiments, purified plasma EVs were directly captured from clarified plasma samples. For example, a diluted sample of purified plasma EVs was incubated with magnetic beads conjugated with respective antibodies for an appropriate time period at an appropriate

temperature, *e.g.*, at room temperature. In some embodiments, purified plasma EVs were mixed with antibody-conjugated magnetic beads during incubation.

Binding of antibody-oligonucleotide conjugates to EVs bound on magnetic capture beads:

[0590] Antibody-oligonucleotide conjugates were diluted in an appropriate buffer at their optimal concentrations. Antibody probes were allowed to interact with a sample comprising EVs bound on magnetic capture beads.

Post-binding washes:

[0591] In some embodiments, samples were washed, *e.g.*, multiple times, in an appropriate buffer.

Ligation:

[0592] After the wash to remove unbound antibody-oligonucleotide conjugates, the beads with bound extracellular vesicles and bound antibody-oligonucleotide conjugates were contacted with a ligation mix. The mixtures were then incubated for 20 minutes at room temperature (RT).

PCR:

[0593] Following ligation, the beads with bound extracellular vesicles and bound antibody-oligonucleotide conjugates were contacted with a PCR mix. PCR were performed in a 96-well plate, *e.g.*, on the Quant Studio 3, with the following exemplary PCR protocol: hold at 95 °C for 1 minute, perform 50 cycles of 95 °C for 5 seconds and 62 °C for 15 seconds. The rate of temperature change was chosen to be standard (*e.g.*, 2 °C per second). A single qPCR reaction was performed for each experimental replicate and ROX was as the passive reference to normalize the qPCR signals. Data were then downloaded from the Quant Studio 3 machine and analyzed and plotted in Python 3.7.

Data analysis:

[0594] In some embodiments, a binary classification system can be used for data analysis. In some embodiments, signals from a detection assay may be normalized based on a reference signal. For example, in some embodiments, normalized signals for a single antibody duplex was calculated by choosing a reference sample. In some embodiments, the equations used to calculate the normalized signal for an arbitrary sample *i* are given below, where $Signal_{max}$ is the signal from the highest concentration cell-line EVs standard.

$$\Delta Ct_i = Ct_{ref} - Ct_i$$

$$Signal_i = 2^{\Delta Ct_i}$$

$$Norm\ Signal_i = \frac{Signal_i}{Signal_{max}}$$

[0595] The present Example describes the use of biomarker combinations (*e.g.*, as described herein) in the assay described in **Figs. 1** and **2** (*e.g.* the biomarkers used in combination with a duplex assay). The assay may be capable of detecting ovarian cancer with at least 99% specificity and/or at least 70% sensitivity. In some embodiments, a biomarker combination includes

targets of capture and detection probes. In some embodiments, use of two or more biomarker combinations in an assay may increase the specificity and/or sensitivity of the assay.

[0596] In some embodiments, a dendron, which can add up to 16 strands of oligonucleotide domain (*e.g.*, DNA) per antibody, can be used instead of one or two strands of DNA per antibody, for example, to enhance signal-to-noise.

Example 2: Embodiments of ovarian cancer detection

[0597] The present Example describes combinations of target biomarkers for ovarian cancer detection (*e.g.*, HGSOC). Samples were handled, processed, and analyzed as described in assays herein (*e.g.*, one as described in Example 1).

[0598] Extracellular vesicles were obtained from plasma samples of subjects belonging to one of the following groups: healthy females, late stage HGSOC high CA-125 levels pool 1, late stage HGSOC high CA-125 levels pool 2, late stage HGSOC low CA-125 levels pool, positive control cell line, and negative control (no extracellular vesicles). In some embodiments, subject samples were pooled together with other subject samples that have been purchased from a particular vendor.

[0599] In some embodiments, the group of healthy female subjects have no history of cancer. In some embodiments, plasma samples from such subjects display CA-125 plasma levels within the normal range (< 25 U/mL CA-125).

[0600] In some embodiments, subjects with late stage HGSOC and high CA-125 plasma level have been diagnosed with stage 3 (*e.g.*, 3A, 3B, or 3C) or stage 4 HGSOC. In some embodiments, such subjects have been diagnosed with grade 3 tumors. In some embodiments, plasma samples from such subjects display CA-125 plasma levels greater than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 plasma levels in the range of 25 U/mL to 3,000 U/mL CA-125.

[0601] In some embodiments, subjects with late stage HGSOC and low CA-125 plasma level have been diagnosed with stage 3 (*e.g.*, 3A, 3B, or 3C) or stage 4 HGSOC. In some embodiments, such have been diagnosed with grade 2 or 3 tumors. In some embodiments, plasma samples from such subjects display CA-125 plasma levels less than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 plasma levels in the range of 0.5 U/mL to 25 U/mL CA-125. In some embodiments, ~20% of ovarian cancer cases have plasma CA-125 values within the normal range.

[0602] In some embodiments, biomarker combinations were evaluated in assays as described herein (*e.g.*, one as described in Example 1). In some embodiments, biomarker combinations were evaluated for their sensitivity to detect ovarian cancer in subject samples versus samples from healthy subjects. In some embodiments, biomarker combinations were evaluated for

their sensitivity to detect ovarian cancer in subject samples versus both samples from healthy subjects and samples from subjects with benign tumors. In some embodiments, biomarker combinations were further evaluated by applying a minimum healthy Ct cutoff of 29 or 32.

Table 3 biomarker combinations for detection of ovarian cancer and corresponding sensitivity relative to control samples. A skilled artisan reading the present disclosure will understand that targets of “Detection Probe 1” and “Detection Probe 2” in a given combination can be used interchangeably. Targets of capture probes described herein are also referred to as capture biomarkers, while targets of detection probes are also referred to as detection biomarkers.

Target of Capture Probe	Target of Detection Probe 1	Target of Detection Probe 2	Sensitivity vs. Healthy at 100% Specificity	Sensitivity vs. Healthy/Benign at 100% Specificity
MUC16	FOLR1	MUC16	0.643 (95% CI: 0.480-0.784)	0.357 (95% CI: 0.216-0.520)
SLC34A2	FOLR1	FOLR1	0.293 (95% CI: 0.161-0.455)	0.293 (95% CI: 0.161-0.455)
SLC34A2	MUC16	MUC16	0.293 (95% CI: 0.161-0.455)	0.293 (95% CI: 0.161-0.455)
BST2	FOLR1	FOLR1	0.500 (95% CI: 0.342-0.658)	0.310 (95% CI: 0.176-0.471)
CA19-9	BST2	BST2	0.190 (95% CI: 0.086-0.341)	0.190 (95% CI: 0.086-0.341)
CA19-9	BST2	MUC16	0.214 (95% CI: 0.103-0.368)	0.214 (95% CI: 0.103-0.368)
CA19-9	CLDN3	CLDN3	0.048 (95% CI: 0.006-0.162)	0.048 (95% CI: 0.006-0.162)
CA19-9	SLC34A2	SLC34A2	0.048 (95% CI: 0.006-0.162)	0.048 (95% CI: 0.006-0.162)
MUC1	BCAM	BCAM	0.214 (95% CI: 0.103-0.368)	0.214 (95% CI: 0.103-0.368)
MUC1	BCAM	BST2	0.293 (95% CI: 0.161-0.455)	0.293 (95% CI: 0.161-0.455)
MUC1	BST2	BST2	0.714 (95% CI: 0.554-0.843)	0.714 (95% CI: 0.554-0.843)
MUC1	BST2	FOLR1	0.786 (95% CI: 0.632-0.897)	0.643 (95% CI: 0.480-0.784)
MUC1	BST2	MUC1	0.690 (95% CI: 0.529-0.824)	0.667 (95% CI: 0.505-0.804)
MUC1	BST2	sTn	0.342 (95% CI: 0.196-0.514)	0.342 (95% CI: 0.196-0.514)
MUC1	BST2	sTn	0.366 (95% CI: 0.221-0.531)	0.366 (95% CI: 0.221-0.531)
MUC1	MSLN	MUC1	0.368 (95% CI: 0.218-0.540)	0.368 (95% CI: 0.218-0.540)
MUC1	MSLN	sTn	0.381 (95% CI: 0.236-0.544)	0.381 (95% CI: 0.236-0.544)
MUC1	sTn	sTn	0.619 (95% CI: 0.456-0.764)	0.405 (95% CI: 0.256-0.567)

Target of Capture Probe	Target of Detection Probe 1	Target of Detection Probe 2	Sensitivity vs. Healthy at 100% Specificity	Sensitivity vs. Healthy/Benign at 100% Specificity
MUC16	BCAM	BCAM	0.195 (95% CI: 0.088-0.349)	0.171 (95% CI: 0.072-0.321)
MUC16	FOLR1	FOLR1	0.667 (95% CI: 0.505-0.804)	0.452 (95% CI: 0.298-0.613)
MUC16	FOLR1	SLC34A2	0.619 (95% CI: 0.456-0.764)	0.405 (95% CI: 0.256-0.567)
MUC16	MUC1	MUC1	0.786 (95% CI: 0.632-0.897)	0.405 (95% CI: 0.256-0.567)
MUC16	MUC1	MUC16	0.452 (95% CI: 0.298-0.613)	0.095 (95% CI: 0.027-0.226)
MUC16	MUC1	sTn	0.452 (95% CI: 0.298-0.613)	0.357 (95% CI: 0.216-0.520)
MUC16	MUC16	sTn	0.548 (95% CI: 0.387-0.702)	0.310 (95% CI: 0.176-0.471)
MUC16	MSLN	sTn	0.158 (95% CI: 0.060-0.313)	0.158 (95% CI: 0.060-0.313)
MUC16	MSLN	sTn	0.390 (95% CI: 0.242-0.555)	0.390 (95% CI: 0.242-0.555)
MUC16	SLC34A2	SLC34A2	0.048 (95% CI: 0.006-0.162)	0.048 (95% CI: 0.006-0.162)
MUC16	SLC34A2	sTn	0.119 (95% CI: 0.040-0.256)	0.119 (95% CI: 0.040-0.256)
MUC16	sTn	sTn	0.784 (95% CI: 0.618-0.902)	0.378 (95% CI: 0.225-0.552)
MUC16	sTn	sTn	0.714 (95% CI: 0.554-0.843)	0.310 (95% CI: 0.176-0.471)
sTn	BST2	MUC1	0.500 (95% CI: 0.334-0.666)	0.500 (95% CI: 0.334-0.666)
sTn	FOLR1	MUC16	0.789 (95% CI: 0.627-0.904)	0.632 (95% CI: 0.460-0.782)
sTn	FOLR1	MSLN	0.526 (95% CI: 0.358-0.690)	0.526 (95% CI: 0.358-0.690)
sTn	MUC1	MSLN	0.526 (95% CI: 0.358-0.690)	0.500 (95% CI: 0.334-0.666)
sTn	MUC16	MSLN	0.658 (95% CI: 0.486-0.804)	0.421 (95% CI: 0.263-0.592)
sTn	BST2	MUC1	0.690 (95% CI: 0.529-0.824)	0.690 (95% CI: 0.529-0.824)
sTn	FOLR1	MUC1	0.737 (95% CI: 0.569-0.866)	0.684 (95% CI: 0.513-0.825)
sTn	FOLR1	MUC16	0.810 (95% CI: 0.659-0.914)	0.643 (95% CI: 0.480-0.784)
sTn	FOLR1	MSLN	0.667 (95% CI: 0.505-0.804)	0.595 (95% CI: 0.433-0.744)
sTn	FOLR1	FOLR1	0.690 (95% CI: 0.529-0.824)	0.619 (95% CI: 0.456-0.764)
sTn	MUC1	MSLN	0.738 (95% CI: 0.580-0.861)	0.548 (95% CI: 0.387-0.702)
sTn	MUC1	SLC34A2	0.762 (95% CI: 0.605-0.879)	0.310(95% CI: 0.176-0.471)

Target of Capture Probe	Target of Detection Probe 1	Target of Detection Probe 2	Sensitivity vs. Healthy at 100% Specificity	Sensitivity vs. Healthy/Benign at 100% Specificity
sTn	MUC16	cleaved MUC16	0.452 (95% CI: 0.298-0.613)	0.214 (95% CI: 0.103-0.368)
sTn	MUC16	MSLN	0.690 (95% CI: 0.529-0.824)	0.381 (95% CI: 0.236-0.544)
sTn	cleaved MUC16	MSLN	0.167 (95% CI: 0.070-0.314)	0.167 (95% CI: 0.070-0.314)
sTn	FOLR1	SLC34A2	0.238 (95% CI: 0.121-0.395)	0.071 (95% CI: 0.015-0.195)
T	BST2	BST2	0.333 (95% CI: 0.196-0.495)	0.286 (95% CI: 0.157-0.446)

[0603] *Figs. 7-15* shows performance of certain biomarker combinations identified in Table 3 above.

[0604] In some embodiments, a biomarker combination including SLC34A2 and FOLR1 (*e.g.*, in some embodiments, SLC34A2 capture (*e.g.*, SLC34A2 immunoaffinity capture with FOLR1 + FOLR1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, HGSOC patients).

[0605] In some embodiments, a biomarker combination including SLC34A2 and MUC16 (*e.g.*, in some embodiments, SLC34A2 capture (*e.g.*, SLC34A2 immunoaffinity capture with MUC16 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, HGSOC patients).

[0606] In some embodiments, a biomarker combination including MUC16 and FOLR1 (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with FOLR1 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, HGSOC patients).

[0607] In some embodiments, certain biomarker combinations were selected based on overall average delta Ct across 3 pools of subject samples from late stage HGSOC cancer patients. *Fig. 9* shows performance of such certain biomarker combinations.

[0608] In some embodiments, a biomarker combination including sTn antigen and BST2 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with BST2 + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late stage HGSOC patients).

[0609] In some embodiments, a biomarker combination including MUC16 and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with MUC16 + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late stage HGSOc patients).

[0610] In some embodiments, a biomarker combination including sTn antigen and FOLR1 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with FOLR1 + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0611] In some embodiments, a biomarker combination including sTn antigen and FOLR1 and MUC16 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with FOLR1 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0612] In some embodiments, a biomarker combination including sTn antigen and MSLN and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with MSLN + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0613] In some embodiments, a biomarker combination including MUC16 and SLC34A2 and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with SLC34A2 + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0614] In some embodiments, a biomarker combination including MUC16 and sTn antigen and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with sTn antigen + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0615] In some embodiments, a biomarker combination including sTn antigen and FOLR1 and FOLR1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with FOLR1 + FOLR1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOc patients).

[0616] In some embodiments, a biomarker combination including MUC1 and BST2 and BST2 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOC patients).

[0617] In some embodiments, a biomarker combination including MUC16 and MUC1 and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with MUC1 + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, late-stage HGSOC patients).

[0618] In some embodiments, certain biomarker combinations were selected based on the average delta Ct from samples of late stage HGSOC cancer patients with low CA-125 plasma levels. *Fig. 10* shows performance of such certain biomarker combinations.

[0619] In some embodiments, a biomarker combination including MUC16 and MUC1 (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with MUC1 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0620] In some embodiments, a biomarker combination including CA19-9 antigen and SLC34A2 (*e.g.*, in some embodiments, CA19-9 antigen capture (*e.g.*, CA19-9 antigen immunoaffinity capture with SLC34A2 + SLC34A2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0621] In some embodiments, a biomarker combination including T antigen and BST2 (*e.g.*, in some embodiments, T antigen capture (*e.g.*, T antigen immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0622] In some embodiments, a biomarker combination including sTn antigen and MUC16 and cleaved MUC16 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with MUC16 + cleaved MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in

Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0623] In some embodiments, a biomarker combination including sTn antigen and MSLN and MUC16 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with MSLN + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0624] In some embodiments, a biomarker combination including CA19-9 antigen and BST2 and MUC16 (*e.g.*, in some embodiments, CA19-9 antigen capture (*e.g.*, CA19-9 antigen immunoaffinity capture with BST2 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

[0625] In some embodiments, a biomarker combination including MUC1 and BCAM (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture with BCAM + BCAM antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients with low CA-125 plasma level such as, for example, late-stage HGSOC cancer patients with low CA-125 plasma level).

Example 3: Additional characterization of certain biomarker combinations for ovarian cancer detection

[0626] The present Example describes combinations of target biomarker for ovarian cancer detection (*e.g.*, HGSOC). Samples were handled, processed, and analyzed as described in assays herein (*e.g.*, one as described in Example 1).

[0627] Extracellular vesicles were obtained from plasma samples of subjects belonging to one of the following groups: healthy females, benign ovarian masses, late stage HGSOC, early stage HGSOC, positive control cell line, and negative control (no extracellular vesicles).

[0628] In some embodiments, the group of healthy female subjects have no history of cancer. In some embodiments, plasma samples from such subjects display CA-125 plasma levels in normal range (< 25 U/mL CA-125).

[0629] In some embodiments, subjects with benign ovarian masses have been diagnosed with a mass including, but not limited to, endometrial cyst, serous papillary cystadenoma, cavernous hemangioma, mature teratoma, thecoma, serous cystadenoma, serous cysts, follicular cyst, or fibrotecoma. In some embodiments, plasma samples from such subjects display CA-125 plasma

levels greater than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 25 U/mL to 3,000 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels less than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 0.5 U/mL to 25 U/mL CA-125.

[0630] In some embodiments, subjects with early stage HGSOC have been diagnosed with stage 1 (*e.g.*, 1A, 1B, or 1C) or stage 2 (*e.g.*, 2A, 2B, or 2C) HGSOC. In some embodiments, such subjects have been diagnosed with grade 2, 3, or 4 tumors. In some embodiments, plasma samples from such subjects display CA-125 levels greater than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 25 U/mL to 3,000 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels less than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 0.5 U/mL to 25 U/mL CA-125.

[0631] In some embodiments, subjects with late stage HGSOC have been diagnosed with stage 3 (*e.g.*, 3A, 3B, or 3C) or stage 4 HGSOC. In some embodiments, such subjects have been diagnosed with grade 2, 3, or 4 tumors. In some embodiments, plasma samples from such subjects display CA-125 levels greater than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 25 U/mL to 3,000 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels less than 25 U/mL CA-125. In some embodiments, plasma samples from such subjects display CA-125 levels in the range of 0.5 U/mL to 25 U/mL CA-125.

[0632] In some embodiments, biomarker combinations were evaluated in assays as described herein (*e.g.*, one as described in Example 1). In some embodiments, biomarker combinations were evaluated for their sensitivity to detect ovarian cancer in subject samples versus samples from healthy subjects. In some embodiments, biomarker combinations were evaluated for their sensitivity to detect ovarian cancer in subject samples versus both samples from healthy subjects and samples from subjects with benign tumors.

[0633] *Figs. 16-19* shows performance of certain biomarker combinations.

[0634] In some embodiments, a biomarker combination including MUC16 and FOLR1 (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with FOLR1 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late-stage HGSOC patients).

[0635] In some embodiments, a biomarker combination including sTn antigen and BST2 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with BST2 + MUC1 antibody probes) can be particularly useful for detection of ovarian

cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late-stage HGSOc patients).

[0636] In some embodiments, a biomarker combination including sTn antigen and FOLR1 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with FOLR1 + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOc patients).

[0637] In some embodiments, a biomarker combination including MUC16 and sTn antigen and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with sTn antigen + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOc patients).

[0638] In some embodiments, a biomarker combination including MUC1 and BST2 and BST2 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, ones as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOc patients).

[0639] In some embodiments, a biomarker combination including sTn antigen and MSLN and MUC16 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with MSLN + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOc patients).

[0640] In some embodiments, a biomarker combination including CA19-9 antigen and BST2 (*e.g.*, in some embodiments, CA19-9 antigen capture (*e.g.*, CA19-9 antigen immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOc patients).

[0641] In some embodiments, a biomarker combination including MUC1 and BST2 and FOLR1 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture with BST2 + FOLR1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following

an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, ovarian cancer patients such as, for example, early- or late stage-HGSOC patients).

Example 4: Assessment of extracellular vesicle (EV) surface proteins as ovarian cancer biomarkers

[0642] In some embodiments, ovarian cancer detection includes detection of at least EV surface biomarker(s) following immunoaffinity capture of extracellular vesicles.

[0643] In some embodiments, one or more surface biomarkers or extracellular membrane biomarkers that are present on extracellular vesicles (“capture biomarkers”) can be used for immunoaffinity capture of ovarian cancer-associated extracellular vesicles. Examples of such capture biomarkers may include, but are not limited to (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof.

[0644] In some embodiments, EV immunoassay methodology (*e.g.*, ones described herein such as in Example 1) and biomarker-validation process (*e.g.*, ones described herein such as in Example 1) can be used to assess additional surface biomarkers as biomarkers for ovarian cancer. In some embodiments, an antibody directed to a capture biomarker (*e.g.*, a surface biomarker present on ovarian cancer-associated EVs) is conjugated to magnetic beads and evaluated, optionally first on cell-line EVs then on patient samples, for its ability to bind the specific target biomarker. The antibody-coated bead is assessed for its ability to capture ovarian cancer-associated EVs and the captured EVs by the antibody-coated bead is read out using a target entity detection system (*e.g.*, a duplex system as described herein involving a set of two detection probes (*e.g.*, as described herein), each directed to a target marker that is distinct from the capture biomarker.

[0645] In some embodiments, captured EVs can be read out using at least one (*e.g.*, 1, 2, 3, or more) surface biomarker, which is or comprises (i) at least one intact or cleaved polypeptide encoded by a human gene as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof. In some embodiments, captured EVs can be read out using a set of detection probes (*e.g.*, as utilized and/or described herein), at least two of which are directed to one or more (*e.g.*, 1, 2, 3, or more) surface biomarkers, which are or comprise (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof. In some embodiments, a polypeptide

encoded by human gene *MUC16* is an intact MUC16 polypeptide. In some embodiments, polypeptide encoded by human gene *MUC16* is a cleaved MUC16 polypeptide. In some embodiments, a set of detection probes comprises two detection probes each directed to the same surface biomarker. In some embodiments, a set of detection probes comprises two detection probes each directed to a distinct surface biomarker.

Example 5: Assessment of mRNA in extracellular vesicles (intravesicular mRNA) as ovarian cancer biomarkers

[0646] In some embodiments, ovarian cancer detection includes detection of at least intravesicular mRNA(s) following immunoaffinity capture of extracellular vesicles.

[0647] In some embodiments, one or more surface biomarkers or extracellular membrane biomarkers that are present on extracellular vesicles (“capture biomarkers”) can be used for immunoaffinity capture of ovarian cancer-associated extracellular vesicles. Examples of such capture protein biomarkers may include but are not limited to polypeptides encoded by human genes as described in Example 4 and carbohydrate markers as described in Example 4.

[0648] In some embodiments, EV nucleic acid detection assay (*e.g.*, reverse transcription PCR using primer-probe sets) and biomarker-validation process (*e.g.*, ones described herein such as in Example 1) can be used to assess mRNA biomarker candidates for ovarian cancer. In some embodiments, an antibody directed to a capture biomarker (*e.g.*, a surface biomarker present in ovarian cancer-associated EVs) is conjugated to magnetic beads and evaluated, optionally first on cell-line EVs then on patient samples, for its ability to bind the specific target biomarker. The antibody-coated bead is assessed for its ability to capture ovarian cancer-associated EVs and the captured EVs by the antibody-coated bead is profiled for their mRNA contents, for example, using one-step quantitative reverse transcription PCR (RT-qPCR) master mix.

[0649] In some embodiments, captured EVs can be read out by detection of at least one or more (*e.g.*, 1, 2, 3, or more) of the following mRNAs: CRABP2, MIF, CLDN6, PRAME, S100A1, KLK7, and combinations thereof. In some embodiments, captured EVs can be read out by detection of one or more (*e.g.*, 1, 2, 3, or more) of the following mRNAs using RT-qPCR: CRABP2, MIF, CLDN6, PRAME, S100A1, KLK7, and combinations thereof.

[0650] In some embodiments, captured EVs can be read out by detection of at least one or more (*e.g.*, 1, 2, 3, or more) intravesicular RNA biomarkers, *e.g.*, CRABP2, MIF, CLDN6, PRAME, S100A1, KLK7, and combinations thereof; and at least one or more (*e.g.*, 1, 2, 3, or more) of EV surface proteins described in Example 4. For example, in some embodiments, an intravesicular RNA biomarker may be or comprise an mRNA transcript encoded by a human gene described herein. In some embodiments, an intravesicular RNA biomarker may be or comprise a microRNA. In some embodiments, an intravesicular RNA biomarker may be or comprise long noncoding RNA. In some

embodiments, an intravesicular RNA biomarker may be or comprise piwi-interacting RNA. In some embodiments, an intravesicular RNA biomarker may be or comprise circular RNA. In some embodiments, an intravesicular RNA biomarker may be or comprise small nucleolar RNA. In some embodiments, an intravesicular RNA biomarker may be or comprise an orphan noncoding RNA.

[0651] In some such embodiments, captured EVs can be read out (i) by detection of one or more (*e.g.*, 1, 2, 3, or more) of the following mRNAs using RT-qPCR: CRABP2, MIF, CLDN6, PRAME, S100A1, KLK7, and combinations thereof; and (ii) by using a set of detection probes (*e.g.*, as utilized and/or described herein), at least one of which are directed to one or more (*e.g.*, 1, 2, 3, or more) of EV surface proteins described in Example 4 (“surface biomarker detection”). In some embodiments, intravesicular biomarker detection is performed after surface biomarker detection. For example, in some embodiments, captured EVs after intravesicular biomarker detection can be contacted with a lysing agent to release intravascular analytes (including, *e.g.*, intravesicular RNA biomarkers) for detection and analysis.

[0652] In some embodiments for surface biomarker detection, a set of detection probes comprises at least one detection probe directed to an EV surface biomarker. In some such embodiments, a set of detection probes comprises at least two detection probes directed to the same EV surface biomarker (with the same or different epitopes). In some such embodiments, a set of detection probes comprises at least two detection probes directed to distinct EV surface biomarkers. In some such embodiments, a set of detection probes comprises at least one detection probe directed to an EV surface biomarker. In some embodiments, a set of detection probes comprises at least two detection probes directed to the same EV surface biomarker (with the same or different epitopes). In some embodiments, a set of detection probes comprises at least two detection probes directed to distinct EV surface biomarkers.

[0653] In some embodiments, a sample comprising an EV surface biomarker and intravesicular mRNA can be contacted with an anti-EV surface biomarker affinity agent (*e.g.*, an antibody directed to EV surface biomarker as described in Example 4) conjugated to a single-stranded oligonucleotide (*e.g.*, DNA) that serves as one of two primers in a pair for an intravesicular mRNA biomarker (*e.g.*, as described herein) such that the anti-EV surface biomarker affinity agent is bound to the EV surface biomarker while the conjugated single-stranded oligonucleotide is hybridized with the intravesicular mRNA biomarker present in the same sample. A second primer of the pair and an RT-qPCR probe are then added to perform an RT-qPCR for detection of the presence of an intravesicular mRNA and an EV surface biomarker in a single sample.

[0654] The present Example further demonstrates exemplary methods for detection of at least one (*e.g.*, 1, 2, 3, or more) intravesicular RNA biomarker in extracellular vesicles derived from cancer cell lines. In some embodiments, such a method comprises immunoaffinity capture of extracellular vesicles as described herein (*e.g.*, via a surface-bound protein such as a surface

biomarker described herein), followed by detection of intravesicular RNA, for example, by reverse-transcription qPCR (RT-qPCR). In some embodiments, extracellular vesicles are captured by a cancer-associated surface biomarker, e.g., in some embodiments using antibody-functionalized solid substrate (e.g., magnetic beads). In some embodiments, captured extracellular vesicles are lysed to release their nucleic acid cargo prior to detection of intravesicular RNA. In some embodiments, intravesicular RNA is or comprises mRNA.

[0655] In some embodiments, cell lines were selected that originate from or are associated with cancer (e.g., a particular cancer type). In some embodiments, such cell lines were selected that originate from or are associated with colon/colorectal cancer, leukemia, melanoma, ovarian cancer, or sarcoma (e.g., rhabdoid tumor). In some embodiments, G-401, K562, NIH:OVCAR-3, SK-MEL-1, or T84 cell lines were selected.

[0656] In some embodiments, extracellular vesicles were purified from conditioned cell culture medium, counted, immunoaffinity captured, and washed via methods as described herein (e.g., as described in Example 1).

[0657] Each RT-qPCR reaction mixture included a PCR reaction mixture (e.g., 50% (volume) Luna One-Step reaction mix, 5% (volume) Luna WarmStart RT enzyme mix, 5% (volume) primer-TaqMan probe mixture), and a variable combination of water, captured extracellular vesicles, and lysing agent. RT-qPCR was performed, for example, on the Quant Studio 3, with a suitable PCR protocol, e.g., hold at 55°C for 10 minutes, hold at 95°C for 1 minute, perform 50 cycles of 95°C for 5 seconds and 62°C for 15 seconds, and standard melt curve. The rate of temperature change was chosen to be standard (2°C per second). All qPCRs were performed in doublets or triplets and ROX was used as the passive reference to normalize the qPCR signals. Data was then downloaded from the Quant Studio 3 machine and analyzed and plotted in Python 3.7. Primers and TaqMan probes for each gene were purchased from Integrated DNA Technologies (IDT) as a 20X concentrate.

[0658] As an initial experiment, *MIF* mRNA was found to be detected in 5e7 bulk extracellular vesicles that were lysed with 1% IGEPAL. Table I shows *MIF* expression in transcript per million (TPM) from different cell lines.

[0659] **Table I: Summary of *MIF* mRNA expression for cancer cell lines.** *MIF* RT-PCR signal (45-Ct) for a panel of bulk cell line EVs with varying gene expression. Samples were tested in singlicate and 5e7 EVs were used per reaction.

Cell Line	MIF Expression (TPM)
SK-MEL-1	1302.5
NIH:OVCAR-3	1175.3
T84	196.8
G-401	0.8

[0660] A similar experiment was performed to further demonstrate this approach across different intravesicular RNA biomarkers. Table II shows mRNA transcript expression levels in 5e7

bulk extracellular vesicles from different cell lines and shows that mRNA is detectable in cell-line EVs at levels that are dependent on cell gene expression.

[0661] **Table II: Summary of expression of four different mRNA transcripts for cancer cell lines**

mRNA transcript	Cell Line	Expression (TPM)	RT-PCR signal of 5e7 lysed EVs	
			Replicate 1	Replicate 2
CLDN6	OVCAR-3	203.4	34.0	33.9
	SK-MEL-1	0.1	40.5	Undetected
	No template control	-	Undetected	Undetected
FAM83A	K-562	220.1	29.9	29.7
	G-401	0.1	Undetected	Undetected
	No template control	-	Undetected	Undetected
HMGB3	NCI-H1781	759.5	27.4	27.3
	OVCAR-3	293.6	37.4	37.5
	No template control	-	Undetected	Undetected
B3GNT3	NCI-H1781	134.9	33.6	33.9
	G-401	0	Undetected	Undetected
	No template control	-	Undetected	Undetected

[0662] Additionally, an experiment was performed to detect the colocalization of at least one intravesicular RNA biomarkers with at least one surface biomarker (e.g., a surface marker that is associated with extracellular vesicles). In some embodiments, extracellular vesicles are captured using antibody-functionalized beads directed to a surface biomarker that is present on the surface of the extracellular vesicles. For example, in the present Example, EPCAM-targeted beads were used to capture extracellular vesicles. Bound extracellular vesicles were lysed and *MIF* mRNA content was quantified via RT-qPCR. In some embodiments, a positive control cell line is selected that expresses a surface biomarker for capture and/or an intravesicular RNA biomarker for detection (e.g., EPCAM+, MIF+), while a negative control cell line is selected that does not express a target biomarker for capture and/or detection (e.g., EPCAM-, MIF+). NIH:OVCAR-3 was selected as a positive control cell line and SK-MEL-1 was selected as a negative control cell line. Multiple detergent conditions were also assessed in this experiment to assess the effect of detergent (e.g., Tween-20) concentration on assay performance. These data indicate that reducing detergent (e.g., Tween-20) concentration can improve assay performance. Without wishing to be bound by a particular theory, this effect may likely be due to preservation of membrane integrity during extracellular vesicle capture, as Tween-20 may permeabilize membranes.

[0663] The present Example demonstrates that intravesicular RNA can be detected via RT-qPCR. In particular, the present Example demonstrates that colocalization of surface biomarkers

and intravesicular RNA in extracellular vesicles can be detected by immunoaffinity capture via a surface biomarker followed by RT-qPCR analysis of intravascular RNA.

Example 6: Assessment of intravesicular biomarkers as ovarian cancer biomarkers

[0664] In some embodiments, ovarian cancer detection includes detection of at least intravesicular protein(s) following immunoaffinity capture of extracellular vesicles.

[0665] In some embodiments, one or more surface proteins or extracellular membrane biomarkers that are present on extracellular vesicles (“capture biomarkers”) can be used for immunoaffinity capture of ovarian cancer-associated extracellular vesicles. Examples of such capture biomarkers may include, but are not limited to polypeptides encoded by human genes as described in Example 4 and carbohydrate biomarkers as described in Example 4.

[0666] In some embodiments, EV immunoassay methodology (*e.g.*, ones described herein such as in Example 1) and biomarker-validation process (*e.g.*, ones described herein such as in Example 1) can be used to assess intravesicular proteins as biomarkers for ovarian cancer. In some embodiments, an antibody directed to a capture biomarker (*e.g.*, a surface protein present in ovarian cancer-associated EVs) is conjugated to magnetic beads and evaluated, first on cell-line EVs then on patient samples, for its ability to bind the specific target protein biomarker. The antibody-coated bead is assessed for its ability to capture ovarian cancer-associated EVs and the captured EVs by the antibody-coated beads are fixed and/or permeabilized prior to being profiled for their intravesicular proteins using a target entity detection system (*e.g.*, a duplex system as described herein involving a set of two detection probes, each directed to a target marker that is distinct from the capture protein).

[0667] In some embodiments, captured EVs after fixation and/or permeabilization can be read out using at least one or more (*e.g.*, 1, 2, 3, or more) of the following intravesicular proteins: CRABP2, KLK7, MIF, PRAME, and S100A1, and combinations thereof. In some embodiments, captured EVs after fixation and/or permeabilization can be read out using a set of detection probes (*e.g.*, as utilized and/or described herein), at least two of which are directed to one or more (*e.g.*, 1, 2, 3, or more) intravesicular biomarkers (*e.g.*, as described herein). In some embodiments, a set of detection probes comprises two detection probes each directed to the same intravesicular biomarker (*e.g.*, as described herein). In some embodiments, a set of detection probes comprises two detection probes each directed to a distinct intravesicular biomarker.

[0668] In some embodiments, captured EVs after fixation and/or permeabilization can be read out using (i) at least one (*e.g.*, 1, 2, 3, or more) intravesicular biomarkers (*e.g.*, as described herein); and (ii) at least one (*e.g.*, 1, 2, 3, or more) EV surface biomarkers described in Example 4. In some embodiments, captured EVs after fixation and/or permeabilization can be read out using a set of detection probes (*e.g.*, as utilized and/or described herein), which comprises (i) a first detection probe directed to one or more (*e.g.*, 1, 2, 3, or more) intravesicular biomarkers (*e.g.*, as described

herein); and (ii) a second detection probe directed to one or more (*e.g.*, 1, 2, 3, or more) of EV surface biomarkers described in Example 4.

Example 7: Development of an ovarian cancer liquid biopsy assay

[0669] The present Example describes development of an ovarian cancer liquid biopsy assay, for example, for screening hereditary- and average-risk individuals. Despite being the fifth largest killer of women among all cancers (Howlander *et al.*, 2019; which is incorporated herein by reference for the purpose described herein), there is currently no recommended ovarian cancer screening tool for average-risk women. This is due, in part, to the poor performance of proposed ovarian cancer screening technologies. Given the incidence of ovarian cancer in average-risk women, inadequate test specificities (<99.5%) result in false positive results that outnumber true positives by more than an order of magnitude. This places a significant burden on the healthcare system and on the women being screened as false positive results lead to additional tests, unnecessary surgeries, and emotional/physical distress (Buys *et al.*, 2011; which is incorporated herein by reference for the purpose described herein). As a result, it may be desirable to develop a non-invasive ovarian cancer screening test from blood that may exhibit two features to provide clinical utility: (1) ultrahigh specificity (>99.5%) to minimize the number of false positives, and (2) high sensitivity (>40%) for stage I and II ovarian cancer when prognosis is most favorable. The development of such a test has the potential to save tens of thousands of lives each year.

[0670] Several different biomarker classes have been studied for an ovarian cancer liquid biopsy assay including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), bulk proteins, and extracellular vesicles (EVs). EVs are particularly promising due to their abundance and stability in the bloodstream relative to ctDNA and CTCs, suggesting improved sensitivity for early-stage cancers. Moreover, EVs contain cargo (*e.g.*, proteins, RNA, metabolites) that originated from the same cell, providing superior specificity over bulk protein measurements. While the diagnostic utility EVs has been studied, much of this work has pertained to bulk EV measurements or low-throughput single-EV analyses.

[0671] This present Example describes one aspect of an exemplary approach for early stage ovarian cancer detection through the profiling of individual extracellular vesicles (EVs) in human plasma. EVs, including exosomes and microvesicles, contain co-localized proteins, RNAs, metabolites, and other compounds representative of their cell of origin (Kosaka *et al.*, 2019; which is incorporated herein by reference for the purpose described herein). The detection of strategically chosen co-localized markers within a single EV can enable the identification of cell type with ultrahigh specificity, including the ability to distinguish cancer cells from normal tissues. As opposed to other cancer diagnostic approaches that rely on cell death for biomarkers to enter the blood (*i.e.*, cfDNA), EVs are released at a high rate by functioning cells. Single cells have been shown to release

as many as 10,000 EVs per day *in vitro* (Balaj *et al.*, 2011; which is incorporated herein by reference for the purpose described herein). In addition, it is widely accepted that cancer cells release EVs at a higher rate than healthy cells (Bebelman *et al.* 2018; which is incorporated herein by reference for the purpose described herein).

[0672] In one aspect, the present disclosure provides insights and technologies involving identification of genes that are upregulated in ovarian cancer versus healthy tissues using Applicant's proprietary bioinformatic biomarker discovery process. From a list of upregulated biomarkers, biomarker combinations that are predicted to exhibit high sensitivity and specificity for ovarian cancer are designed. Using an exemplary individual EV assay (see, *e.g.*, illustrated in *Fig. 1 or 2* and/or described herein), co-localization of such biomarkers on an individual vesicle is detected, indicating that the grouping of biomarkers originated from the same cell. This provides superior specificity to bulk biomarker measurements, including bulk EV assays, given that many upregulated cancer biomarkers, such as MUC16, are expressed by one or more healthy tissues. Through the careful design of biomarker combinations, signals from competing tissues can be reduced or eliminated, including those closely related to ovarian cancer. In some embodiments, the present disclosure provides technologies with ultrahigh specificity that is particularly helpful as an ovarian cancer screening test for which the prevalence of disease is low and a high positive-predictive value (>10%) is required (Seltzer *et al.*, 1995; which is incorporated herein by reference for the purpose described herein).

Biomarker Discovery

[0673] In some embodiments, a biomarker discovery process leverages bioinformatic analysis of large databases and an understanding of the biology of ovarian cancer and extracellular vesicles.

Individual Extracellular Vesicle Analysis

[0674] The detection of tumor-derived EVs in the blood requires an assay that has sufficient selectivity and sensitivity to detect relatively few tumor-derived EVs per milliliter of plasma in a background of 10 billion EVs from a diverse range of healthy tissues. The present disclosure, among other things, provides technologies that address this challenge. For example, in some embodiments, an assay for individual extracellular vesicle analysis is illustrated in *Fig. 1*, which is performed in three key steps as outlined below:

1. EVs are purified from patient plasma using size-exclusion chromatography (SEC), which removes greater than 99% of soluble proteins and other interfering compounds.
2. Tumor-specific EVs are captured using antibody-functionalized magnetic beads specific to a membrane-associated surface biomarker.

3. A modified version of proximity-ligation-immuno quantitative polymerase chain reaction (pliq-PCR) is performed to determine the co-localization of additional biomarkers contained on or within the captured EVs.

[0675] In many embodiments of a modified version of a pliq-PCR assay, two or more different antibody-oligonucleotide conjugates are added to the EVs captured by the antibody-functionalized magnetic bead and the antibodies subsequently bind to their biomarker targets. The oligonucleotides are composed of dsDNA with single-stranded overhangs that are complementary, and thus, capable of hybridizing when in close proximity (*i.e.*, when the corresponding biomarker targets are located on the same EV). After washing away unbound antibody-oligonucleotide species, adjacently bound antibody-oligonucleotide species are ligated using a standard DNA ligase reaction. Subsequent qPCR of the ligated template strands enables the detection and relative quantification of co-localized biomarker species. In some embodiments, two to twenty distinct antibody-oligonucleotide probes can be incorporated into such an assay, *e.g.*, as described in U.S. Application No. 16/805,637 (issued as US11,085,089), and International Application PCT/US2020/020529 (published as WO2020180741), both filed February 28, 2020 and entitled “Systems, Compositions, and Methods for Target Entity Detection”; which are both incorporated herein by reference in their entirety for any purpose.

[0676] pliq-PCR has numerous advantages over other technologies to profile EVs. For example, pliq-PCR has a sensitivity three orders of magnitude greater than other standard immunoassays, such as ELISAs (Darmanis *et al.*, 2010; which is incorporated herein by reference for the purpose described herein). The ultra-low LOD of a well-optimized pliq-PCR reaction enables detection of trace levels of tumor-derived EVs, down to a thousand EVs per mL. This compares favorably with other emerging EV analysis technologies, including the Nanoplasmic Exosome (nPLEX) Sensor (Im *et al.*, 2014; which is incorporated herein by reference for the purpose described herein) and the Integrated Magnetic-Electrochemical Exosome (iMEX) Sensor (Jeong *et al.*, 2016; which is incorporated herein by reference for the purpose described herein), which have reported LODs of $\sim 10^3$ and $\sim 10^4$ EVs, respectively (Shao *et al.*, 2018; which is incorporated herein by reference for the purpose described herein). Moreover, in some embodiments, a modified version of pliq-PCR approach does not require complicated equipment and can uniquely detect the co-localization of multiple biomarkers on individual EVs.

[0677] In some embodiments, to further improve the sensitivity and specificity of an individual EV profiling assay, other classes of EV biomarkers include mRNA and intravesicular proteins (in addition to EV surface biomarker) can be identified and included in an assay.

Exemplary workflow

[0678] A workflow was developed in which biomarker candidates are validated to be present in EVs and capable of being detected by commercially available antibodies or mRNA primer-probe sets. For a given biomarker of interest, one or more cell lines expressing (positive control) and not expressing the biomarker of interest (negative control) can be cultured to harvest their EVs through concentrating their cell culture media and performing purification to isolate nanoparticles having a size range of interest (*e.g.*, using SEC). Typically, extracellular vesicles may range from 30 nm to several micrometers in diameter. See, *e.g.*, Chuo *et al.*, “Imaging extracellular vesicles: current and emerging methods” *Journal of Biomedical Sciences* 25: 91 (2018) which is incorporated herein by reference for the purpose described herein, which provides information of sizes for different extracellular vesicle (EV) subtypes: migrasomes (0.5-3 μ m), microvesicles (0.1-1 μ m), oncosomes (1-10 μ m), exomeres (<50 nm), small exosomes (60-80 nm), and large exosomes (90-120 nm). In some embodiments, nanoparticles having a size range of about 30 nm to 1000 nm may be isolated for detection assay. In some embodiments, specific EV subtype(s) may be isolated for detection assay.

[0679] Through a proprietary biomarker discovery process, surface biomarkers (*e.g.*, as described herein) that are upregulated in HGSOV versus healthy tissues were identified and used in ovarian cancer patient samples.

[0680] To further improve the performance of an exemplary single EV profile assay (*e.g.*, ones described herein) for detection of ovarian cancer, in some embodiments, additional biomarker candidates including surface biomarkers and intravesicular mRNAs/proteins can be identified.

[0681] In some embodiments, it was previously demonstrated by Applicant the feasibility of EV-mRNA detection using purified cell-line EVs in bulk. Through immunoaffinity capture of a membrane bound protein marker, this approach enables the detection of two co-localized biomarkers. Moreover, EV-mRNA detection requires a simpler protocol because RT-qPCR can be performed directly after immunoaffinity capture. In some embodiments, mRNA detection using EVs can be performed by capturing EVs using capture probes (*e.g.*, as described herein) and detecting a particular ovarian cancer mRNA biomarker. EVs that express both capture probe marker and ovarian cancer mRNA biomarker are selectively detected.

Example 8: Further characterization of exemplary ovarian cancer liquid biopsy assays

[0682] This example is directed towards further characterization of various biomarker combinations in exemplary ovarian cancer liquid biopsy assays using different cell populations and patient populations. Useful biomarker combinations for detection of ovarian cancer can be determined by screening various combinations of biomarkers disclosed herein (*e.g.*, surface biomarkers such as, *e.g.*, (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, and combinations thereof; and/or (ii)

carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and combinations thereof) in pooled control and patient plasma samples. Pooled samples can provide an estimation for the average assay signal among various patient cohorts, facilitating biomarker combination triage.

[0683] In some embodiments, a biomarker combination for detection of ovarian cancer can comprise one, two, three, four, five, six, seven or more biomarkers (*e.g.*, ones described herein), wherein such a combination comprises at least one biomarker for capturing extracellular vesicles (*e.g.*, by immunoaffinity capture) and at least one biomarker (including, *e.g.*, one, two, three, four, five, six, seven, or more biomarkers) for detection by pliq-PCR assay. In some embodiments, a target biomarker for capturing extracellular vesicles (*e.g.*, immunoaffinity capture) can be same as at least one target biomarker for pliq-PCR based analysis. In some embodiments, target biomarkers for capturing extracellular vesicles and pliq-PCR based analysis can each be distinct.

[0684] Various biomarker combinations can be validated in pooled patient cohorts. Patient cohorts can include appropriate patient and/or control populations. In certain embodiments, a patient cohort can comprise post-menopausal healthy women (*e.g.*, healthy women aged between 55 and 79 years of age), pre-menopausal healthy women (*e.g.*, women aged between 20 and 54 years of age), women with advanced HGSOC (*e.g.*, stage III and stage IV HGSOC cases), women with early stage HGSOC (*e.g.*, stage I and stage II HGSOC cases), women with benign gynecological tumors (*e.g.*, women with benign gynecological growths), and/or women with inflammatory diseases, disorders, or conditions (*e.g.*, women with inflammatory conditions including endometriosis, pelvic inflammatory disease, inflammatory bowel disease (*e.g.*, Crohn's disease and/or ulcerative colitis, *etc.*), and/or other inflammatory diseases).

[0685] In some embodiments, a two-step screening procedure can characterize performance of various biomarker combinations. For example, various biomarker combinations (*e.g.*, various combinations of biomarkers for a capture probe (*e.g.*, for immunoaffinity capture) and detection probes) can initially be screened in healthy background pools (from various age groups) and an advanced stage HGSOC pool. In some embodiments, biomarker combinations that exhibit poor separation between the healthy and ovarian cancer pools (*e.g.*, a ΔC_t less than 4, corresponding to less than a 16-fold difference) can be eliminated from further study as a biomarker combination to use in isolation, however, such biomarker combinations may be useful when combined with additional biomarker combinations as described herein. In some embodiments, biomarker combinations that exhibit poor separation between the healthy and ovarian cancer pools (*e.g.*, a ΔC_t less than 2, corresponding to less than a 4-fold difference) can be eliminated from further study as a biomarker combination to use in isolation, however, such biomarker combinations may be useful when combined with additional biomarker combinations as described herein. In some embodiments, biomarker combinations that exhibit poor separation between the healthy and ovarian cancer pools

(*e.g.*, a ΔC_t less than 1, corresponding to less than a 2-fold difference) can be eliminated from further study as a biomarker combination to use in isolation, however, such biomarker combinations may be useful when combined with additional biomarker combinations as described herein.

[0686] In some embodiments, a two-step screening procedure can characterize performance of various biomarker combinations. For example, various biomarker combinations (*e.g.*, various combinations of biomarkers for a capture probe (*e.g.*, for immunoaffinity capture) and detection probes) can initially be screened in healthy background pools (from various age groups) and an advanced stage HGSOC pool. In some embodiments, biomarker combinations that exhibit good diagnostic performance (*e.g.*, a ΔC_t greater than 1, corresponding to greater than a 2-fold difference) can undergo a second round of screening with pooled early stage HGSOC, benign gynecological tumors, and/or inflammatory conditions. In some embodiments, biomarker combinations that exhibit good diagnostic performance (*e.g.*, a ΔC_t greater than 2, corresponding to greater than a 4-fold difference) can undergo a second round of screening with pooled early stage HGSOC, benign gynecological tumors, and/or inflammatory conditions. In some embodiments, biomarker combinations that exhibit good diagnostic performance (*e.g.*, a ΔC_t greater than 4, corresponding to greater than a 16-fold difference) can undergo a second round of screening with pooled early stage HGSOC, benign gynecological tumors, and/or inflammatory conditions. In some embodiments, top biomarker combinations (*e.g.*, the best performing approximately 20 biomarker combinations) that can distinguish early and late stage HGSOC from the control pools can be further evaluated.

[0687] Incorporation of one or more additional biomarker combinations in an exemplary assay (*e.g.*, ones described herein) can improve its sensitivity. In some embodiments, by utilizing at least two biomarker combinations, improved performance of an assay (*e.g.*, at least approximately 80% sensitivity at 98% specificity; or at least approximately 70% sensitivity at 99.5% specificity; or at least approximately 70% sensitivity at 99% specificity) can be achieved. Pooled patient samples can approximate the assay signals across individual patient populations, a trait which can provide a realistic matrix to assess a large number of biomarker combinations in an efficient manner. The top biomarker combinations (*e.g.*, up to 20 biomarker combinations) identified can be further tested in individual patient plasma-based pilot studies. In some embodiments, an individual patient plasma-based pilot study can comprise control patients who are either pre- or post- menopausal, control patients who are asymptomatic, control patients who are symptomatic, control patients with benign gynecological tumors, and/or control patients with non-ovarian cancer inflammatory health conditions. In some embodiments, an individual patient plasma-based pilot study can comprise test patients who are either pre- or post- menopausal, test patients who are symptomatic, test patients who are asymptomatic, test patients with stage I or stage II HGSOC, and/or test patients with stage III or stage IV HGSOC. Non-HGSOC health conditions can be aged-matched to the ovarian cancer

cohort. It can be expected that the control samples (*e.g.*, healthy controls, benign gynecological tumors, and/or other off-target conditions) can provide an estimate of a log-normal distribution to set signal cutoffs pertaining to a 98% specificity (with approximately 95% CI: 95.3% to 100%) for hereditary risk screening assays and 99.5% specificity (with approximately 95% CI: 98.1% to 100%) for symptomatic triaging assays. Biomarker combination performance characteristics can be evaluated using bivariate associations between combinations to assess independence, and top biomarker combinations can be further tested using a three-variable logistic regression model for predicting ovarian cancer.

[0688] The identification of novel biomarker combinations that can distinguish HGSOC cases from controls and identify orthogonality between combinations can be achieved. This orthogonality can aid in distinguishing HGSOC cases from the control cohorts, increasing the sensitivity of exemplary assays as described herein. To assess racial diversity in the patient cohort, samples can be obtained from vendors which source from diverse populations. In certain embodiments, exemplary biomarker combinations may be specific to particular racial and/or ethnic groups. In certain embodiments, testing exemplary biomarker combinations in diverse racial and/or ethnic groups can identify biomarker combinations of appropriate diagnostic value for specific racial and/or ethnic groups.

[0689] Validation of additional ovarian cancer biomarker combinations in primary patient samples can improve the diagnostic performance of exemplary assays. Through the incorporation of additional, orthogonal biomarker combinations, assay performance can improve beyond 80% sensitivity at 98% specificity and 70% sensitivity at 99.5% specificity and 70% sensitivity and 99% specificity.

Example 9: Validation of exemplary ovarian cancer liquid biopsy assays

[0690] This example relates to the validation of exemplary ovarian cancer liquid biopsy assays in additional populations/cohorts. Independent validation studies to assess exemplary ovarian cancer diagnostic assays as described herein using additional cohorts of patient samples can be performed. Samples can be obtained from any appropriate source. Technical experts are blinded to sample designations prior to any result analysis, and/or assay results are analyzed by an outside independent technical expert. To ensure sampling from a population representative of the United States, at least approximately 20% of samples can be sourced from non-white ethnicities (United States Census Bureau, 2018), depending on sample availability.

[0691] In certain embodiments, patient cohorts analyzed can include: patients at hereditary risk for ovarian cancer prior to undertaking any risk-reducing operations (*e.g.*, patients with familial cases of ovarian cancer and/or BRCA1 or BRCA2 pathological variant carriers who have not undergone bilateral salpingo-oophorectomy or similar procedures, post-menopausal women with

chronic conditions associated with abdominal pain (*e.g.*, women >54 years of age with chronic inflammatory conditions, patients with benign gynecological tumors, and/or patients with HGSOE (*e.g.*, individuals with stage I/II ovarian cancer, and individuals with stage III/IV ovarian cancer). In certain embodiments, a biomarker combination (*e.g.*, ones described herein) can provide at least an approximately 80% sensitivity (95% CI: 68.7% to 91.3%) at approximately 98% specificity (95% CI: 95.5% to 100%) in women with hereditary risk. In certain embodiments, a biomarker combination (*e.g.*, ones described herein) can provide at least approximately 70% sensitivity (95% CI: 57% to 83%) at approximately 99.5% specificity (95% CI: 98.2% to 100%) for post-menopausal symptomatic women. Moreover, in certain embodiments, exemplary assays can differentiate between women with benign tumors and those with HGSOE, resulting in few false positives.

[0692] In certain embodiments, additional validation studies of exemplary assays as described herein can be conducted utilizing longitudinally collected samples from independent sources. In some embodiments, samples can be obtained or derived from longitudinally collected blood draws (*e.g.*, blood draws collected at temporally distinct time points) from ovarian cancer cases. In addition, blood draws from age-matched controls can be analyzed. Using exemplary logistic regression models for a 98% specificity cutoff, assessments can be made of how many years prior to diagnosis (*e.g.*, however many years prior to ovarian cancer diagnosis blood draws are available from) exemplary assay are capable of detecting ovarian cancer while maintaining an annual specificity of 98%. Assay sensitivity can be calculated as a function of year prior to diagnosis while ensuring specificity is maintained in the control samples at 98%.

[0693] In some embodiments, at least an approximately 80% sensitivity (95% CI: 63.3% to 96.7%) for HGSOE at approximately 98% specificity (95% CI: 93.8% to 100%) for samples collected at the time of diagnosis can be expected. Given that ovarian cancer tumors have been shown to exist as stage I/II disease for four or more years (Brown and Palmer, 2009), exemplary assays can be expected to achieve at least approximately 60% sensitivity (95% CI: 39.5% to 80.5%) for HGSOE two years prior to clinical diagnosis. This would pertain to a PPV of 23.3% in this high-risk patient cohort two years before clinical diagnosis, a PPV far better than the current standard of care for women at hereditary risk.

Example 10: Embodiments of assays described herein for early-stage of ovarian cancer

[0694] The present Example describes combinations of target biomarkers for ovarian cancer detection (*e.g.*, HGSOE). In some embodiments, assays as described herein are useful for distinguishing a benign growth from a malignant tumor in a subject presenting with an adnexal mass. Samples were handled, processed, and analyzed as described in assays herein (*e.g.*, one as described in Example 1).

[0695] Extracellular vesicles were obtained from plasma samples of subjects belonging to one of the following groups: healthy females, females with benign gynecological tumors (*e.g.*, women with benign gynecological growths), females with early stage HGSOE (*e.g.*, stage I and stage II HGSOE cases), and females with late stage HGSOE (*e.g.*, stage III and stage IV HGSOE cases).

[0696] CA-125 plasma levels were measured in the samples from which the extracellular vesicles were obtained. Samples were classified as having normal levels of CA-125 if the level was measured at < 25 U/mL CA-125. Samples were classified as having elevated levels of CA-125 if the level was measured at > 25 U/mL CA-125. Based on the studied samples, 100% of samples from the healthy group had CA-125 levels measured in the normal range. About 69% of samples from the benign group had CA-125 levels measured in the normal range and about 31% of samples from the benign group had CA-125 levels measured in the elevated range. About 28% of samples from the early-stage group (*e.g.*, stage I and stage II HGSOE cases) had CA-125 levels measured in the normal range and about 72% of samples from the early-stage group had CA-125 levels measured in the elevated range. About 21% of samples from the late-stage group (*e.g.*, stage III and stage IV HGSOE cases) had CA-125 levels measured in the normal range and about 79% of samples from the late stage group had CA-125 levels measured in the elevated range.

[0697] In the present Example, biomarker combinations were evaluated in assays as described herein (*e.g.*, one as described in Example 1). In some embodiments, biomarker combinations were evaluated for their sensitivity at a pre-determined specificity to detect ovarian cancer in subject samples versus samples from healthy subjects. In some embodiments, biomarker combinations were evaluated for their sensitivity at a pre-determined specificity to detect ovarian cancer in subject samples versus samples from both healthy subjects and subjects with benign tumors. In the present Example, biomarker combinations were each evaluated by applying a minimum healthy Ct cutoff that was determined at a given specificity (*e.g.*, in some embodiments at 95% specificity).

Table 4 Exemplary biomarker combinations for detection of ovarian cancer and corresponding sensitivity relative to control samples. Each biomarker in the column of “Biomarker combinations” can be utilized as a capture biomarker and/or a detection biomarker. For example, in the 3-biomarker combinations below, one of the biomarkers is utilized as a capture biomarker and two others are utilized as detection biomarkers. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is utilized as both a capture biomarker and a detection biomarker, while the other biomarker is utilized as a detection biomarker. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is a capture biomarker, while the other biomarker is

utilized as a detection biomarker in a pair of detection probes. The Ct cutoff values below were determined at 95% specificity.

Biomarker combinations	Overall Sensitivity HGSOC vs. Healthy at 95% Specificity	Exemplary Ct Cutoff Value	Overall Sensitivity HGSOC vs. Healthy/Benign at 95% Specificity	Exemplary Ct Cutoff Value
MUC1, BST2, FOLR1	0.91 (95% CI: 0.77-0.97)	31	0.69 (95% CI: 0.53-0.82)	28
sTn, MUC16, MSLN	0.91 (95% CI: 0.77-0.97)	29	0.69 (95% CI: 0.53-0.82)	27
sTn, FOLR1, MUC1	0.90 (95% CI: 0.75-0.97)	29	0.89 (95% CI: 0.72-0.96)	28
sTn, BST2, MUC1	0.81 (95% CI: 0.66-0.91)	31	0.81 (95% CI: 0.66-0.91)	31
MUC16, MUC1, sTn	0.83 (95% CI: 0.69-0.93)	34	0.50 (95% CI: 0.34-0.66)	30
CA19-9, BST2	0.33 (95% CI: 0.20-0.50)	29	0.52 (95% CI: 0.36-0.68)	31
MUC1, BST2	0.81 (95% CI: 0.66-0.91)	30	0.79 (95% CI: 0.63-0.90)	29
MUC16, sTn	0.81 (95% CI: 0.66-0.91)	28	0.76 (95% CI: 0.61-0.88)	27

[0698] In some embodiments, a biomarker combination including MUC1, BST2, and FOLR1 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture) with BST2 + FOLR1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0699] In some embodiments, a biomarker combination including sTn antigen, MUC16, and MSLN (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture) with MUC16 + MSLN antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0700] In some embodiments, a biomarker combination including sTn antigen, FOLR1, and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture) with FOLR1 + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0701] In some embodiments, a biomarker combination including sTn antigen and BST2 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity

capture with BST2 + MUC1 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0702] In some embodiments, a biomarker combination including MUC16, FOLR1, and MUC16 (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture) with FOLR1 + MUC16 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0703] In some embodiments, a biomarker combination including CA19-9 antigen and BST2 (*e.g.*, in some embodiments, CA19-9 antigen capture (*e.g.*, CA19-9 antigen immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0704] In some embodiments, a biomarker combination including MUC1 and BST2 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture with BST2 + BST2 antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0705] In some embodiments, a biomarker combination including MUC16 and sTn antigen (*e.g.*, in some embodiments, MUC16 capture (*e.g.*, MUC16 immunoaffinity capture with sTn antigen + sTn antigen antibody probes) can be particularly useful for detection of ovarian cancer (*e.g.*, following an assay as described herein (*e.g.*, one as described in Example 1)) in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0706] *Fig. 20 (A-D)* shows performance of certain biomarker combinations identified in **Table 4** above. *Fig. 20 (E-H)* shows the area under the curve (AUC) for the same biomarker combinations. The measured AUC for such biomarker combinations was within a range from 0.905 to 0.952. Specifically, **Fig. 20 (A, E)** shows data for a biomarker combination including MUC1, BST2, and FOLR1 (*e.g.*, in some embodiments, MUC1 capture (*e.g.*, MUC1 immunoaffinity capture) with BST2 + FOLR1 antibody probes). **Fig. 20 (B, F)** shows data for a biomarker combination including sTn antigen, MUC16, and MSLN (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture) with MUC16 + MSLN antibody probes). **Fig. 20 (C, G)** shows data for a biomarker combination including sTn antigen, FOLR1, and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture) with FOLR1 + MUC1 antibody probes). **Fig. 20 (D, H)** shows data for a biomarker combination including sTn antigen and BST2 and MUC1 (*e.g.*, in some embodiments, sTn antigen capture (*e.g.*, sTn antigen immunoaffinity capture with BST2 + MUC1 antibody probes).

[0707] As shown in *Fig. 20*, such biomarker combinations are particularly useful for detection of early-stage ovarian cancer. In some embodiments, such biomarker combinations are particularly useful for detection of late-stage ovarian cancer. In some embodiments, such biomarker combinations are particularly useful for detection of ovarian cancer in normal healthy subject populations and also in certain subject populations (*e.g.*, patients presenting with adnexal masses).

[0708] In some embodiments, certain biomarker combinations described herein can outperform CA-125 ELISA by having fewer false positives and negatives than CA-125 ELISA. For example, an assay as described herein (*e.g.*, as shown in Example 1) in combination with the biomarker combination of sTn antigen, BST2, and MUC1 is shown to be able to detect HGSOC in 6 out of 11 women (3 early, 3 late stage) with normal plasma CA-125 levels (<25 U/mL) whose corresponding CA-125 scored as false negatives. Such an assay also correctly classified 7 out of 8 women with benign masses with elevated plasma CA-125 (>25 U/mL) whose CA-125 scored as false positives. In some embodiments, a different biomarker combination of sTn, FOLR1, and MUC1 is also shown to outperform plasma CA-125 test when comparing ovarian cancer (*e.g.*, HGSOC) against healthy and benign ovarian tumor samples while comparable performance between the assay described herein and the plasma CA-125 test was observed when comparing ovarian cancer (*e.g.*, HGSOC) against healthy patient samples. See *Fig. 21*. By reducing false positives and negatives, women with cancer can get the care they need sooner, and women who are cancer free can be spared the stress and downstream work ups arising from false positive CA-125 assay results.

[0709] These data demonstrate the ability of assays as described herein (together with biomarker combinations as described herein) to detect ovarian cancer (*e.g.*, HGSOC) at an early stage using combinations of 2-3 biomarkers co-localized on the surface of single extracellular vesicles. In some embodiments, biomarker combinations described herein had a sensitivity of 83.3% for ovarian cancer (*e.g.*, HGSOC) stage I/II versus healthy controls and a sensitivity of 90.5% for all ovarian cancer (*e.g.*, HGSOC) versus healthy controls at 95% specificity. Assays as described herein also had fewer false positives and fewer false negatives than conventional plasma CA-125 ELISA. In some embodiments, the data presented herein can be used to form a training model to a larger study using plasma samples from women with early and late ovarian cancer (*e.g.*, HGSOC), benign adnexal masses, healthy controls, inflammatory conditions, and off-target cancers

Example 11: Detection of ovarian cancer in patients with adnexal masses

[0710] The present Example describes certain biomarker combinations that are particularly useful for distinguishing a benign growth from a malignant tumor in a subject presenting with an adnexal mass, and/or from distinguishing ovarian cancer from other off-target cancer types and/or inflammatory conditions. Samples were handled, processed, and analyzed as described in assays

herein (*e.g.*, one as described in Example 1).

Table 5 shows exemplary biomarker combinations for detection of ovarian cancer and corresponding univariate area under the curve (AUC) when discriminating normal and benign samples against early-stage (I/II) HGSOE. Each biomarker in the column of “Biomarker combinations” can be utilized as a capture biomarker and/or a detection biomarker. For example, in the 3-biomarker combinations below, one of the biomarkers is utilized as a capture biomarker and two others are utilized as detection biomarkers. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is utilized as both a capture biomarker and a detection biomarker, while the other biomarker is utilized as a detection biomarker. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is a capture biomarker, while the other biomarker is utilized as a detection biomarker in a pair of detection probes.

Biomarker combinations	Area Under the Curve (AUC)	95% Confidence Intervals (CI)
CA19-9, BST2	0.92	0.86 – 0.97
MUC1, BST2	0.97	0.94 – 1.00
MUC1, BST2, FOLR1	0.96	0.92 – 1.00
MUC16, sTn	0.97	0.94 – 1.00
sTn, BST2, MUC1	0.97	0.93 – 1.00
sTn, FOLR1, MUC1	0.97	0.93 – 1.00
sTn, MUC16, MSLN	0.97	0.93 – 1.00
SLC34A2, FOLR1	0.87	0.80 – 0.94
SLC34A2, MUC16	0.90	0.84 – 0.96
MUC16, FOLR1	0.96	0.91 – 1.00

[0711] The area under the curve (AUC) and corresponding confidence intervals (CI) for biomarker combinations are identified in **Table 5** above. **Figs. 22-23** show the performance of biomarker combinations identified in **Table 5** above and **Fig. 24** shows the performance of a plasma CA-125 ELISA test. Overall, the biomarker combinations identified in **Table 5** above have a clear separation of benign and healthy versus cancer plasma samples, whereas plasma CA-125 has high overlap between benign samples and early-stage HGSOE.

Table 6 shows the overall sensitivity of certain biomarker combinations for detection of ovarian cancer at 99% specificity. Each biomarker in the column of “Biomarker combinations” can be utilized as a capture biomarker and/or a detection biomarker. For example, in the 3-biomarker combinations below, one of the biomarkers is utilized as a capture biomarker and two others are

utilized as detection biomarkers. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is utilized as both a capture biomarker and a detection biomarker, while the other biomarker is utilized as a detection biomarker. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is a capture biomarker, while the other biomarker is utilized as a detection biomarker in a pair of detection probes.

Biomarker combinations	Overall Sensitivity HGSOC vs. Healthy at 99% Specificity	Overall Sensitivity HGSOC vs. Healthy/Benign at 99% Specificity
CA19-9, BST2	0.73 (95% CI:0.63-0.82)	0.461 (95% CI: 0.35-0.57)
MUC1, BST2	0.88 (95% CI:0.79-0.94)	0.69 (95% CI: 0.58-0.78)
MUC1, BST2, FOLR1	0.78 (95% CI: 0.68-0.86)	0.62 (95% CI: 0.51-0.72)
MUC16, sTn	0.93 (95% CI: 0.86-0.98)	0.72 (95% CI: 0.61-0.81)
sTn, BST2, MUC1	0.91 (95% CI:0.83-0.96)	0.82 (95% CI: 0.73-0.89)
sTn, FOLR1, MUC1	0.80 (95% CI: 0.70-0.88)	0.79 (95% CI: 0.69-0.87)
sTn, MUC16, MSLN	0.83 (95% CI: 0.73-0.90)	0.72 (95% CI: 0.61-0.81)
SLC34A2, FOLR1	0.48 (95% CI: 0.37-0.59)	0.25 (95% CI: 0.16-0.35)
SLC34A2, MUC16	0.60 (95% CI: 0.48-0.70)	0.44 (95% CI: 0.33-0.55)
MUC16, FOLR1	0.74 (95% CI: 0.63-0.82)	0.48 (95% CI: 0.37-0.59)

Table 7 shows the overall sensitivity of biomarker combinations for detection of ovarian cancer by stage at 99% specificity. Each biomarker in the column of “Biomarker combinations” can be utilized as a capture biomarker and/or a detection biomarker. For example, in the 3-biomarker combinations below, one of the biomarkers is utilized as a capture biomarker and two others are utilized as detection biomarkers. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is utilized as both a capture biomarker and a detection biomarker, while the other biomarker is utilized as a detection biomarker. In some embodiments of the 2-biomarker combinations below, one of the biomarkers is a capture biomarker, while the other biomarker is utilized as a detection biomarker in a pair of detection probes.

Biomarker combinations	Stage I Sensitivity HGSOC vs. Healthy at 99% Specificity	Stage II Sensitivity HGSOC vs. Healthy at 99% Specificity	Stage III Sensitivity HGSOC vs. Healthy at 99% Specificity
Number of Samples	17	32	40
CA19-9, BST2	0.59 (95% CI: 0.33-0.82)	0.69 (95% CI: 0.51-0.83)	0.76 (95% CI: 0.64-0.86)
MUC1, BST2	0.77 (95% CI: 0.50-0.93)	0.86 (95% CI: 0.70-0.95)	0.900 (95% CI: 0.81-0.96)

MUC1, BST2, FOLR1	0.59 (95% CI: 0.33-0.82)	0.74 (95% CI: 0.56-0.87)	0.83 (95% CI: 0.71-0.91)
MUC16, sTn	0.82 (95% CI: 0.57-0.96)	0.94 (95% CI: 0.81-0.99)	0.96 (95% CI: 0.88-0.99)
sTn, BST2, MUC1	0.77 (95% CI: 0.50-0.93)	0.89 (95% CI: 0.73-0.97)	0.94 (95% CI: 0.86-0.98)
sTn, FOLR1, MUC1	0.65 (95% CI: 0.38-0.86)	0.71 (95% CI: 0.54-0.85)	0.83 (95% CI: 0.72-0.91)
sTn, MUC16, MSLN	0.71 (95% CI: 0.44-0.90)	0.77 (95% CI: 0.59-0.89)	0.86 (95% CI: 0.75-0.93)
SLC34A2, FOLR1	0.31 (95% CI: 0.11-0.59)	0.43 (95% CI: 0.26-0.61)	0.50 (95% CI: 0.38-0.62)
SLC34A2, MUC16	0.47 (95% CI: 0.23-0.72)	0.53 (95% CI: 0.34-0.72)	0.62 (95% CI: 0.49-0.74)
MUC16, FOLR1	0.59 (95% CI: 0.33-0.82)	0.71 (95% CI: 0.54-0.85)	0.77 (95% CI: 0.65-0.86)

[0712] In addition, performance of assays (*e.g.*, as described in Example 1) using certain biomarker combinations described herein (*e.g.*, as listed in **Table 4** or **Table 5**) to differentiate ovarian cancer from different types of benign adnexal masses was also evaluated. **Fig. 25** depicts data for performance of four specific biomarker combinations, namely **(A: MUC1, BST2, FOLR1; B: sTn, BST2, MUC1; C: sTn, FOLR1, MUC1; and D: sTn, MUC16, MSLN)** to differentiate ovarian cancer (*e.g.*, early-stage and/or late-stage ovarian cancer) from different types of benign adnexal masses including, *e.g.*, adenofibroma, fibroma, ovarian cyst, cystadenoma, endometriosis, leiomyoma, teratoma, cystadenofibroma, *etc.*

[0713] Furthermore, performance of assays (*e.g.*, as described in Example 1) using certain biomarker combinations described herein (*e.g.*, as listed in **Table 4** or **Table 5**) to differentiate ovarian cancer from off-target cancers was also evaluated. **Fig. 26** depicts comparison of a specific biomarker combination (sTn, FOLR1, and MUC1) and a plasma CA-125 ELISA test in the usefulness to differentiate ovarian cancer (*e.g.*, early-stage and/or late-stage ovarian cancer) from off-target cancers. In general, the tested biomarker combination of (sTn, FOLR1, and MUC1) can be used to differentiate certain off-target cancers from ovarian cancers. As seen in **Fig. 26**, a small subset of samples from both uterine and lung cancers seemed to have been picked up by this specific biomarker combination. Based on TCGA data, it is known in the art that certain biomarkers that are up-regulated in ovarian cancer are also up-regulated in lung adenocarcinoma. As for uterine cancer, there is a serous endometrial cancer subtype in uterine that is presumably similar to serous ovarian cancer. Plasma CA-125 has a similar off-target profile with a few more false positives in pancreatic cancer. Interestingly, there was one sample among the breast cancer group that is detected in both assays, which could possibly be a BRCA+ breast cancer case with an actual ovarian tumor or ovarian metastasis.

[0714] Moreover, performance of assays (e.g., as described in Example 1) using certain biomarker combinations described herein (e.g., as listed in **Table 4** or **Table 5**) to differentiate ovarian cancer from certain inflammatory conditions was evaluated. **Fig. 27** depicts comparison of a specific biomarker combination (sTn, BST2, and MUC1) and a plasma CA-125 ELISA test in the usefulness to differentiate ovarian cancer (e.g., early-stage and/or late-stage ovarian cancer) from certain inflammatory conditions. As seen in **Fig. 27**, very little off-target signal was observed among inflammatory conditions.

[0715] Additionally, EV-based assay technologies as described herein (e.g., as described in Example 1) using a set of 7 different biomarker combinations as shown in **Table 8** below were assessed for their usefulness for detection of ovarian cancer. As shown below, in some embodiments, such a set of biomarker combinations is particularly useful for discriminating patients with ovarian cancer (e.g., HGSOC) from patients with benign adnexal masses. In some embodiments, such a set of biomarker combinations is particularly useful for detection of early-stage ovarian cancer (e.g., Stage I and/or Stage II ovarian cancer).

Table 8 Exemplary biomarker combinations used for detection of ovarian cancer

Exemplary biomarker combinations
MUC1, BST2, FOLR1
sTn, MUC16, MSLN
sTn, FOLR1, MUC1
sTn, BST2, MUC1
CA19-9, BST2
MUC1, BST2
MUC16, sTn

[0716] A prediction model was derived on a retrospectively collected cohort of 42 HGSOC and 26 benign ovarian tumor samples from 2 commercial vendors and 24 healthy controls using a Random Forest Classifier. See, for example, Fawagreh *et al.*, "Random forests: from early developments to recent advancements." *Systems Science & Control Engineering: An Open Access Journal* 2.1 (2014); Breiman, "Random forests." *Machine learning* 45.1 (2001); and Breiman, (2002), "Manual On Setting Up, Using, And Understanding Random Forests V3.1", which is available online at stat.berkeley.edu/~breiman/Using_random_forests_V3.1.pdf. The contents of each of the aforementioned references are incorporated herein by reference in their entirety for the purposes described herein. The reproducibility of this model was assessed on an independent cohort of 89 HGSOC (Stage I (n=17), II (n=35), III (n=37)) and 192 benign ovarian tumor samples from tissue banks and 124 healthy controls. Furthermore, 87 samples from women with off-target cancers and 42 samples from women with inflammatory conditions were obtained from vendors and were analyzed in assays as described herein. Plasma CA-125 levels were measured for each sample using a commercial ELISA.

[0717] The prediction model that combined the set of 7 biomarker combinations as listed in **Table 8** above distinguished ovarian cancer, *e.g.*, HGSOC (including both early-stage and late-stage patients) from benign and healthy patients with an AUC of 0.97 (95% CI 0.93-0.99), with 89% (0.80-0.94) sensitivity at 99% specificity (**Fig. 28 (A)**). For early-stage ovarian cancer (*e.g.*, stage I and stage II HGSOC cases), the model achieved an AUC of 0.94 (0.9-0.99), with 83% (0.70-0.92) sensitivity at 99% specificity (**Fig. 28 (B)**). By comparison, plasma CA-125 achieved an AUC of 0.88 (0.81-0.94) and 19% (0.1-0.33) sensitivity at 99% specificity. Direct comparison of plasma CA-125 and a model described herein showed a significant difference at 99% specificity for both all and stage I/II-stage HGSOC (McNemar p-val < 0.001). When comparing healthy samples, there was no apparent difference between the model and plasma CA-125 (p-val = 1.0; **Fig. 29(A)**) and a significant difference when comparing samples from patients with benign ovarian tumors (p-val < 0.001 for stage I/II-stage HGSOC; **Fig. 29(B)**). This tracks well with findings in the medical literature that describe high numbers of false positives among benign ovarian masses, making plasma CA-125 suboptimal for the early detection of ovarian cancer. The prediction model using the combined set of 7 biomarker combinations clearly outperforms plasma CA-125 with a McNemar p-value < 0.001 at 99% specificity. Both the 7 biomarker combinations model and plasma CA-125 had 1 false positive out of 42 inflammatory cases.

[0718] These data indicate that detecting biomarkers co-localized on individual EVs can detect all stages of ovarian cancer (*e.g.*, HGSOC) from plasma samples with higher specificity and sensitivity than plasma CA-125. Assays described herein (including biomarker combinations described herein) may improve on plasma CA-125 by distinguishing stage I/II ovarian cancer (*e.g.*, HGSOC) from benign ovarian tumors and can have clinical utility for both early detection and treatment stratification between benign and malignant ovarian tumors.

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EQUIVALENTS

[0719] Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, *etc.*, from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Further, it should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. The scope of the present

invention is not intended to be limited to the above Description, but rather is as set forth in the claims that follow.

CLAIMS

What is claimed is:

1. A method comprising steps of:
 - (a) providing or obtaining a biological sample comprising nanoparticles having a size within the range of about 30 nm to about 1000 nm, which are isolated from a bodily fluid-derived sample (e.g., a blood-derived sample) of a subject;
 - (b) detecting on surfaces of the nanoparticles co-localization of at least one set of surface biomarkers, wherein the at least one set comprises at least two surface biomarkers whose combined expression level has been determined to be associated with ovarian cancer, wherein the surface biomarkers are selected from: (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof;
 - (c) comparing the detected co-localization level with the determined level; and
 - (d) classifying the subject as having or being susceptible to ovarian cancer when the detected co-localization level is at or above the determined level.
2. The method of claim 1, wherein the step of detecting on surfaces comprises analyzing nanoparticles that have been separated from other components of the sample by affinity capture targeting at least one of the surface biomarkers on their surfaces.
3. The method of claim 1 or 2, wherein the step of detecting on surfaces comprises contacting the nanoparticles with at least one set of detection probes, each directed to at least one of the surface biomarkers, which set comprises at least a first detection probe for a first surface biomarker and a second detection probe for a second surface biomarker, wherein the first surface biomarker and the second surface biomarker is the same or different.
4. The method of claim 3, wherein the first detection probe comprises a first target-binding moiety directed at the first surface biomarker 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 directed at the second surface biomarker 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.

5. The method of claim 4, wherein the first single-stranded overhang and/or the second single-stranded overhang are four nucleotides in length.
6. The method of claim 5, wherein the first single-stranded overhang or the second single-stranded overhang has a nucleotide sequence of GAGT.
7. The method of any one of claims 4-6, wherein the first oligonucleotide domain and the second oligonucleotide domain have a combined length such that, when the first and second surface biomarkers are simultaneously present on the nanoparticles and the probes of the set of detection probes are bound to their respective surface biomarkers on the nanoparticles, the first single-stranded overhang and the second single-stranded overhang can hybridize together, forming a double-stranded complex.
8. The method of claim 7, 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.
9. The method of claim 8, wherein the nucleic acid ligase is or comprises a DNA ligase (e.g., T4 or T7 DNA ligase).
10. The method of claim 3, wherein the first surface biomarker and the second surface biomarker are the same target biomarker.
11. The method of any one of claims 1-10, wherein the step of detecting on surfaces further comprises a step of amplifying a product that is associated with the co-localization, and detecting the presence of the amplified product.
12. The method of claim 11, wherein the step of amplifying is or comprises quantitative polymerase chain reaction.
13. The method of any one of claims 1-12, wherein the step of detecting on surfaces comprises immobilizing nanoparticles on a solid substrate.
14. The method of claim 13, wherein the solid substrate is or comprises a bead.
15. The method of claim 14, wherein the bead is a magnetic bead.
16. The method of claim 13, wherein the solid substrate is or comprises a surface.
17. The method of claim 16, wherein the surface is a capture surface of a filter, a matrix, a membrane, a plate, a tube, and/or a well.
18. The method of any one of claims 1-17, wherein the steps of (b) and (c) are repeated for a plurality of (e.g., at least two, at least three, at least four, at least five, at least six, at least seven, or more) sets of surface biomarkers, each set comprising at least two surface biomarkers whose combined expression level has been determined to be associated with ovarian cancer, wherein the surface biomarkers are selected from: (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-

- dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof.
19. The method of claim 18, further comprising, for each set of surface biomarkers, combining a result from the step of comparing to determine a score.
 20. The method of claim 19, comprising classifying the subject as having or being susceptible to ovarian cancer when the score is determined to be associated with ovarian cancer.
 21. The method of any one of claims 1-20, wherein the surface biomarkers are selected from: (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof.
 22. The method of any one of claims 1-21, wherein the at least one set or at least one of the plurality of sets of surface biomarkers is/are selected from the following combinations:
 - (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
 - (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*; and
 - (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
 - (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
 - (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
 - (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
 - (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.
 23. The method of any one of claims 1-21, wherein the at least one set or at least one of the plurality of sets of surface biomarkers comprise all of the following combinations:
 - (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
 - (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
 - (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
 - (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
 - (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;

- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
 - (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.
24. The method of any one of claims 1-23, wherein the determined level is determined by comparing combined expression level in ovarian cancer-associated extracellular vesicles relative to extracellular vesicles in comparable samples from a population of non-cancer subjects.
 25. The method of claim 24, wherein the population of non-cancer subjects comprises one or more of the following subject populations: healthy subjects, subjects diagnosed with benign tumors, and subjects with non-ovarian-related diseases, disorders, and/or conditions.
 26. The method of any one of claims 1-25, wherein the nanoparticles have a size within the range of about 50 nm to about 500 nm.
 27. The method of any one of claims 1-26, wherein the nanoparticles are or comprise extracellular vesicles.
 28. The method of any one of claims 1-27, wherein the nanoparticles are isolated from a bodily fluid-derived sample (e.g., a blood-derived sample) by a size-exclusion method.
 29. A kit for detection of ovarian cancer comprising:
 - (a) a capture agent comprising a target-capture moiety directed to a first surface biomarker; and
 - (b) at least one set of detection probes, which set comprises at least two detection probes each directed to a second surface biomarker, wherein the detection probes each comprise:
 - (i) a target binding moiety directed at the second surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same nanoparticle having a size within the range of about 30 nm to about 1000 nm;
 wherein at least the first surface biomarker and the second surface biomarker form a target biomarker signature determined to be associated with ovarian cancer, and wherein the first and second surface biomarkers are each independently selected from: (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof.
 30. The kit of claim 29, wherein the first and/or second surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide an intact MUC16 polypeptide.

31. The kit of claim 29, wherein the first and/or second surface biomarker is a polypeptide encoded by the human gene *MUC16*, wherein the polypeptide is a cleaved MUC16 polypeptide.
32. The kit of any one of claims 29-31, wherein the target binding moiety of at least two detection probes is each directed to the same target surface biomarker of the target biomarker signature.
33. The kit of any one of claims 29-33, wherein the oligonucleotide domain of the at least two detection probes are different.
34. The kit of any one of claims 29-31, wherein the target binding moiety of at least two detection probes is each directed to a distinct target surface biomarker of the target biomarker signature.
35. The kit of any one of claims 29-34, further comprising at least one additional reagent (*e.g.*, a ligase, a fixation agent, and/or a permeabilization agent).
36. The kit of any one of claims 29-35, comprising at least two sets (including, *e.g.*, at least three sets) of detection probes, which each set comprises at least two detection probes each directed to a target surface biomarker of a distinct target biomarker signature for ovarian cancer.
37. The kit of any one of claims 29-36, comprising:
 - (a) a first capture agent comprising a target-capture moiety;
 - (b) a second capture agent comprising a target-capture moiety;
 - (c) at least two sets of detection probes, wherein the detection probes each comprise:
 - (i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same nanoparticle.
38. The kit of any one of claims 29-36, comprising:
 - (a) a first capture agent comprising a target-capture moiety;
 - (b) a second capture agent comprising a target-capture moiety;
 - (c) a third capture agent comprising a target-capture moiety;
 - (d) at least three sets of detection probes, wherein the detection probes each comprise:
 - (i) a target binding moiety directed at a target surface biomarker; 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 at least two detection probes are characterized in that they can hybridize to each other when the at least two detection probes are bound to the same nanoparticle.

39. The kit of any one of claims 29-38, wherein the nanoparticle has a size within the range of about 50 nm to about 500 nm.
40. The kit of any one of claims 29-39, wherein the nanoparticle is or comprises an extracellular vesicle (e.g., an exosome).
41. The kit of any one of claims 29-40, wherein the nanoparticle is isolated from a bodily fluid-derived sample (e.g., a blood-derived sample) by a size-exclusion method.
42. A complex comprising:
 - (a) a nanoparticle having a size within the range of about 30 nm to about 1000 nm and comprising at least a first surface biomarker and a second surface biomarker on its surface, which combination is determined to be a target biomarker signature for ovarian cancer, wherein the first surface biomarker and the second surface biomarker are each independently selected from: (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof;
 - (b) a solid substrate comprising a target-capture moiety directed to the first surface biomarker; wherein the target-capture moiety binds to the first surface biomarker of the nanoparticle such that the nanoparticle is immobilized on the solid substrate; and
 - (c) at least a first detection probe and a second detection probe each bound to the nanoparticle, wherein each detection probe comprises:
 - (i) a target binding moiety directed to the second surface biomarker; 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.
43. The complex of claim 42, wherein the first surface biomarker and the second surface biomarker(s) are different.
44. The complex of claim 42 or 43, wherein the first surface biomarker and the second surface biomarker are each independently selected from: (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof.
45. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a polypeptide encoded by human gene *SLC34A2*; and (ii) one or more surface biomarkers, which

include polypeptides encoded by human genes as follows: *FOLR1*, *MUC16*, and combinations thereof.

46. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a polypeptide encoded by human gene *MUC16*; and (ii) one or more surface biomarkers, which include at least one polypeptide encoded by a human gene as follows: *BCAM*, *FOLR1*, *MUC1*, *MUC16*, *MSLN*, *SLC34A2*, or combinations thereof; and/or (ii) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen.
47. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a polypeptide encoded by human gene *BST2*; and (ii) one or more surface biomarkers comprising a polypeptide encoded by human gene *FOLR1*.
48. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a carbohydrate-dependent marker comprising Sialyl Lewis A antigen (also known as CA19-9); and (ii) one or more surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *CLDN3*, *SLC34A2*, or combinations thereof.
49. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a polypeptide encoded by human gene *MUC1*; and (ii) one or more surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *BST2*, *FOLR1*, *MSLN*, *MUC1*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
50. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a polypeptide encoded by human gene *MUC16*; and (ii) one or more surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BCAM*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof; and/or (ii) at least one carbohydrate-dependent marker as follows: SialylTn (sTn) antigen.
51. The complex of any one of claims 42-44, wherein the target biomarker signature comprises (i) a carbohydrate-dependent marker comprising SialylTn (sTn) antigen; and (ii) one or more surface biomarkers, which include at least one polypeptide encoded by human gene as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*, or combinations thereof.
52. The complex of any one of claims 42-51, wherein the target binding moiety of at least two detection probes is each directed to the same target surface biomarker of the target biomarker signature.
53. The complex of claim 52, wherein the oligonucleotide domain of the at least two detection probes are different.
54. The complex of any one of claims 42-51, wherein the target binding moiety of the at least two detection probes is each directed to a distinct target biomarker of the target biomarker signature.
55. The complex of any one of claims 42-54, wherein the solid substrate comprises a magnetic bead.

56. The complex of any one of claims 42-55, wherein the target-capture moiety is or comprises an antibody agent.
57. The complex of any one of claims 42-56, wherein the nanoparticle is or comprises an extracellular vesicle (e.g., exosome).
58. The complex of any one of claims 42-57, wherein the nanoparticle was isolated from a bodily fluid sample (e.g., a blood sample) taken from a subject.
59. The complex of any one of claims 42-58, wherein the nanoparticle was isolated from a subject's bodily fluid sample (e.g., a blood sample) by a size-exclusion method.
60. The complex of claim 58 or 59, wherein the subject is a human subject.
61. The complex of any one of claims 42-60, wherein the formation of the complex is indicative of a ovarian cancer-associated nanoparticle.
62. The complex of any one of claims 42-61, wherein the single-stranded overhang portions of the first and second detection probes are at least partially complementary.
63. The complex of any one of claims 42-62, wherein the nanoparticle has a size within the range of about 50 nm to about 500 nm.
64. A set of probes for use in a method, kit, or complex of any one of claims 1-63, wherein each set of probes comprises: (a) a biomarker binding moiety that specifically binds to a surface biomarker on nanoparticles having a size within the range of about 300 nm to about 1000 nm and found in a cancer subject's sample; 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, wherein the target biomarkers are each independently selected from: (i) polypeptides encoded by human genes as follows: *BCAM*, *BST2*, *CLDN3*, *FOLR1*, *MSLN*, *MUC1*, *MUC16*, *SLC34A2*; and/or (ii) carbohydrate-dependent markers as follows: SialylTn (sTn) antigen, Thomsen-Friedenreich (T, TF) antigen, Tn antigen, Sialyl Lewis A antigen (also known as CA19-9), and/or combinations thereof.
65. A method for differentiating benign adnexal mass from ovarian cancer, wherein the method comprises:
 - (a) detecting, in a blood-derived sample from a female subject determined to have an adnexal mass, on surfaces of nanoparticles having a size within the range of about 300 nm to about 1000 nm co-localization of at least one biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:
 - (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*; and

- (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (iii) combinations thereof;
 - (b) comparing the detected co-localization level with a reference level; and
 - (c) identifying the adnexal mass of the female subject to be likely benign when the detected co-localization level is or comparable to the reference level; or identifying the adnexal mass to be cancerous when the detected co-localization level is above the reference level.
66. The method of claim 65, wherein the nanoparticles are or comprise extracellular vesicles.
67. The method of claim 65 or 66, wherein the method for differentiating benign adnexal mass from ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 65% to 95%.
68. The method of any one of claims 65-67, wherein the female subject is determined to have an elevated serum CA-125 level (*e.g.*, greater than 25 U/mL).
69. A method for detection of early-stage ovarian cancer, wherein the method comprises:
- (a) detecting, in a blood-derived sample from a female subject, on surfaces of nanoparticles having a size within the range of about 300 nm to about 1000 nm co-localization of at least one biomarker combination, which comprises at least one capture biomarker and at least one detection biomarker, where the at least one capture biomarker and the at least one detection biomarker are each independently selected from:
 - (i) polypeptides encoded by human genes as follows: *BST2*, *FOLR1*, *MSLN*, *MUC1*, and *MUC16*;
 - (ii) carbohydrate-dependent markers as follows: Sialyl Tn (sTn) antigen, Sialyl Lewis A antigen (also known as CA19-9), and
 - (iii) combinations thereof;
 - (b) comparing the detected co-localization level with a reference level; and
 - (c) identifying the female subject to be negative for ovarian cancer when the detected co-localization level is or comparable to the reference level; or identifying the female subject as likely to have or be susceptible to ovarian cancer, when the detected co-localization level is above the reference level.
70. The method of claim 69, wherein the nanoparticles are or comprise extracellular vesicles.
71. The method of claim 69 or 70, wherein the method for detection of early-stage ovarian cancer has a specificity within a range of 90% to 100% and sensitivity within a range of 80% to 95%.
72. The method of any one of claims 69-71, wherein the female subject is determined to have a normal plasma or serum CA-125 level (*e.g.*, less than or equal to 25 U/mL).

73. The method of any one of claims 65-72, wherein the detecting comprises detecting on surfaces of the nanoparticles co-localization of the at least one biomarker combination, wherein the at least one biomarker combination is selected from one of the following:

- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen.
- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

74. The method of any one of claims 65-73, wherein the detecting comprises detecting on surfaces of the nanoparticles co-localization of each of the following biomarker combinations:

- (i) a sialyl Lewis A antigen (also known as CA19-9) and a polypeptide encoded by human gene *BST2*;
- (ii) a polypeptide encoded by human gene *MUC1* and a polypeptide encoded by human gene *BST2*;
- (iii) a polypeptide encoded by human gene *MUC16* and a sialyl Tn (sTn) antigen;
- (iv) a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *FOLR1*;
- (v) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *FOLR1*, and a polypeptide encoded by human gene *MUC1*;
- (vi) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *BST2*, and a polypeptide encoded by human gene *MUC1*; and
- (vii) a sialyl Tn (sTn) antigen, a polypeptide encoded by human gene *MUC16*, and a polypeptide encoded by human gene *MSLN*.

75. The method of any one of claims 65-74, wherein the detecting comprises:

- (a) capturing the nanoparticles from the blood-derived sample with a capture probe that selectively interacts with the at least one capture biomarker on the nanoparticles;
- (b) contacting the captured nanoparticles with at least one set of at least two detection probes that each selectively interacts with the at least one detection biomarker on the nanoparticles; and
- (c) detecting a product formed when the at least two detection probes of the set are in sufficiently close proximity on the individual nanoparticles.

76. The method of claim 75, wherein the capture probe comprises a target-capture moiety that binds to the capture biomarker.
77. The method of claim 76, wherein the target-capture moiety is or comprises an antibody agent directed to the capture biomarker.
78. The method of any one of claims 75-77 wherein the capture biomarker is or comprises a sialyl Lewis A antigen (also known as CA19-9), a polypeptide encoded by human gene *MUC1*, a polypeptide encoded by human gene *MUC16*, or a sialyl Tn (sTn) antigen.
79. The method of any one of claims 75-78, wherein the capture probe is or comprises a solid substrate comprising the target-capture moiety conjugated thereto.
80. The method of claim 79, wherein the solid substrate comprises a magnetic bead.
81. The method of any one of claims 75-80, wherein the at least two detection probes each comprise:
 - (i) a target binding moiety directed to one of the at least detection biomarker; 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 characterized in that they can hybridize to each other when the detection probes are bound to the same nanoparticle.
82. The method of any one of claims 75-81, wherein the product was formed when the at least two detection probes of the set are in sufficiently close proximity on the individual nanoparticles such that the single-stranded overhang portions of the at least two detection probes of the set hybridize to each other to form a double-stranded complex.
83. The method of claim 82, wherein the product formed comprises a ligated template upon contacting the double-stranded complex with a nucleic acid ligase.
84. The method of any one of claims 75-83, wherein the target binding moieties of the at least two detection probes are each directed to the same detection biomarker.
85. The method of claim 84, wherein the oligonucleotide domain of the at least two detection probes are different.
86. The method of claim 84 or 85, wherein the same detection biomarker is or comprises a polypeptide encoded by human gene *BST2*.
87. The method of claim 86, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Lewis A antigen (also known as CA19-9) or directed to a polypeptide encoded by human gene *MUC1*.
88. The method of claim 84 or 85, wherein the same detection biomarker is or comprises a sialyl Tn (sTn) antigen.

89. The method of claim 88, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC16*.
90. The method of claim 75-83, wherein the target binding moieties of the at least two detection probes are each directed to a distinct detection biomarker.
91. The method of claim 90, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *FOLR1*.
92. The method of claim 91, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a polypeptide encoded by human gene *MUC1*.
93. The method of claim 90, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *BST2* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
94. The method of claim 90, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *FOLR1* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MUC1*.
95. The method of claim 90, wherein the target binding moiety of a first detection probe is directed to a polypeptide encoded by human gene *MUC16* and the target binding moiety of a second detection probe is directed to a polypeptide encoded by human gene *MSLN*.
96. The method of any one of claims 93-95, wherein the target-capture moiety of the capture agent is or comprises at least one antibody agent directed to a sialyl Tn (sTn) antigen.

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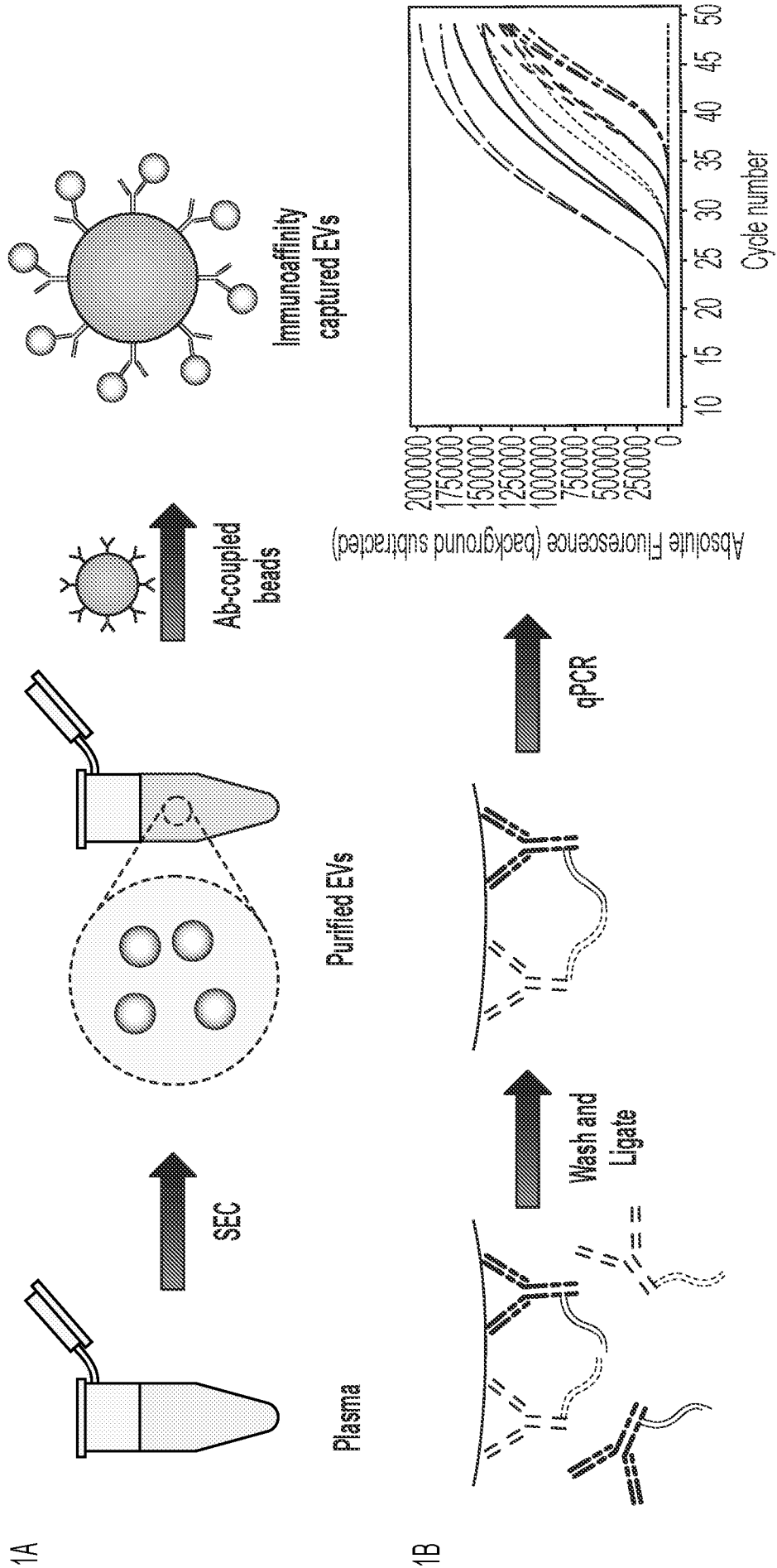


FIG. 1

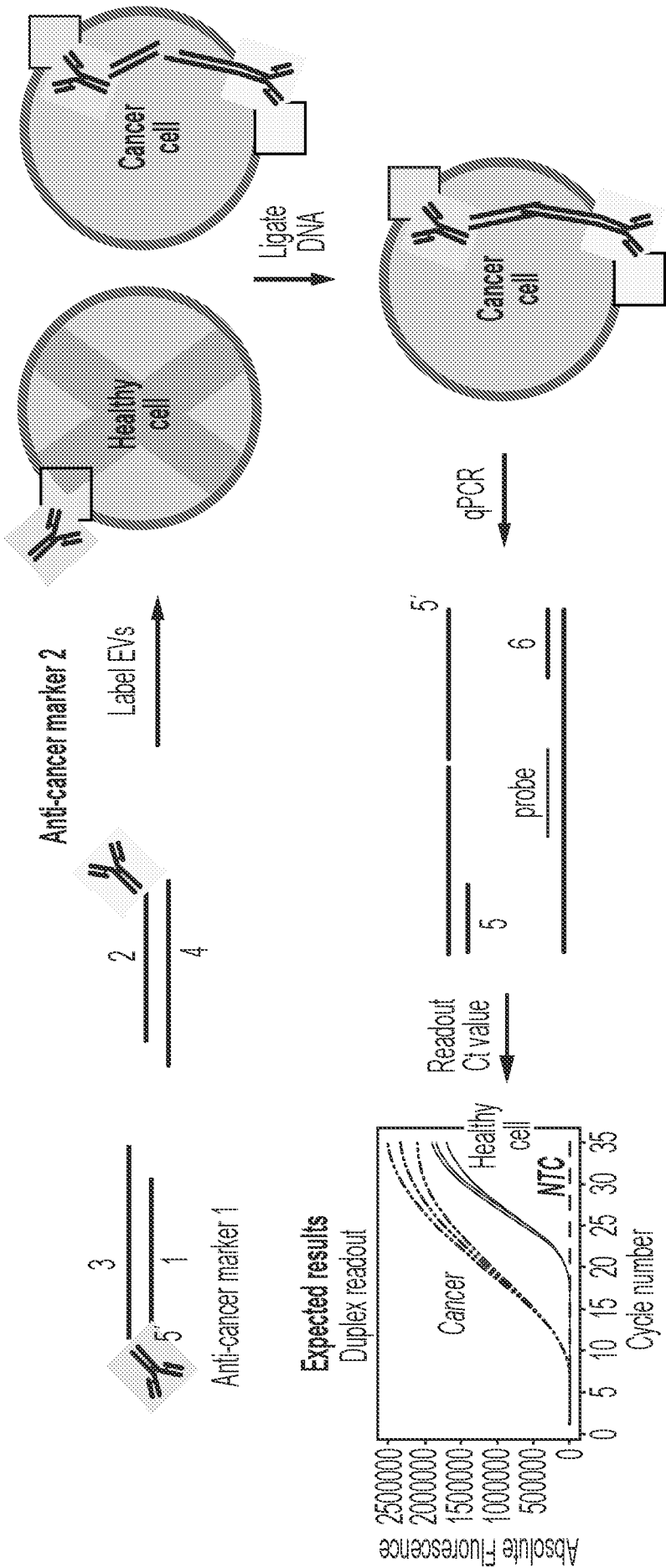


FIG. 2

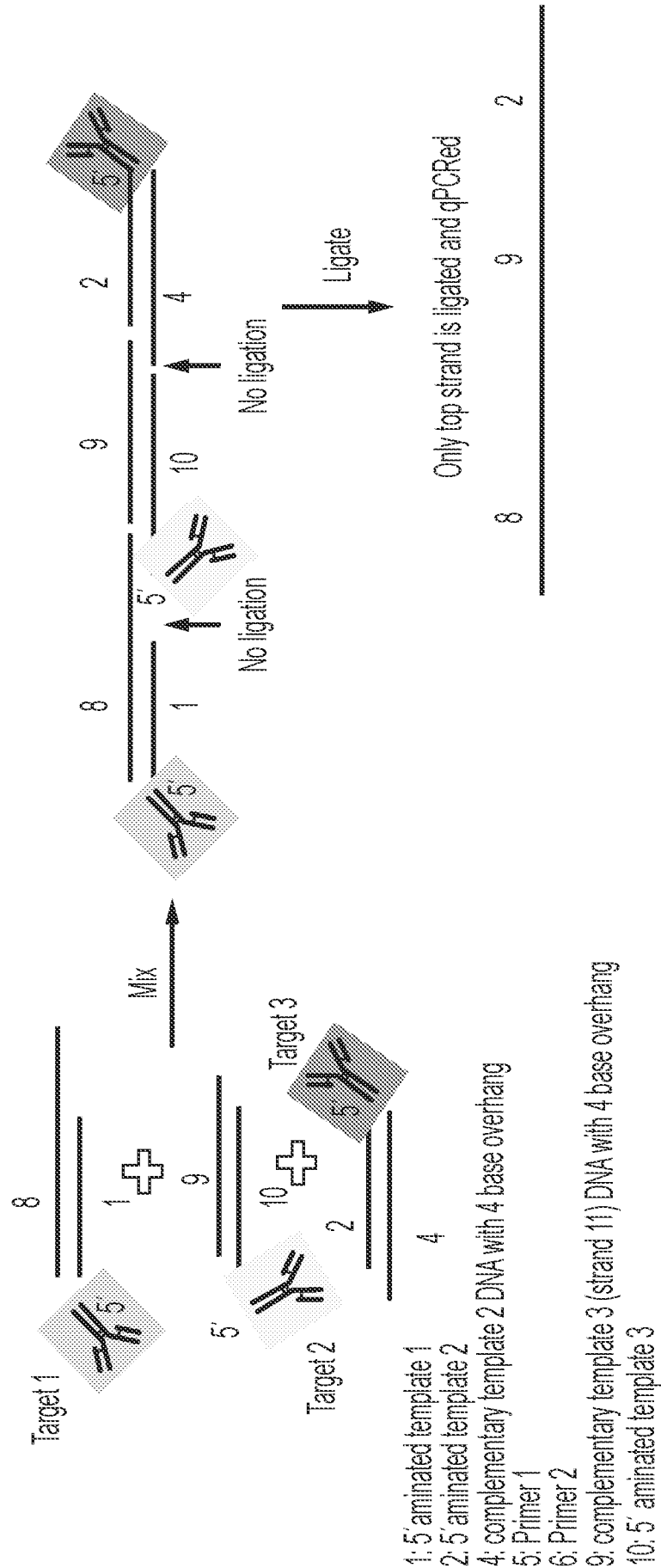


FIG. 3

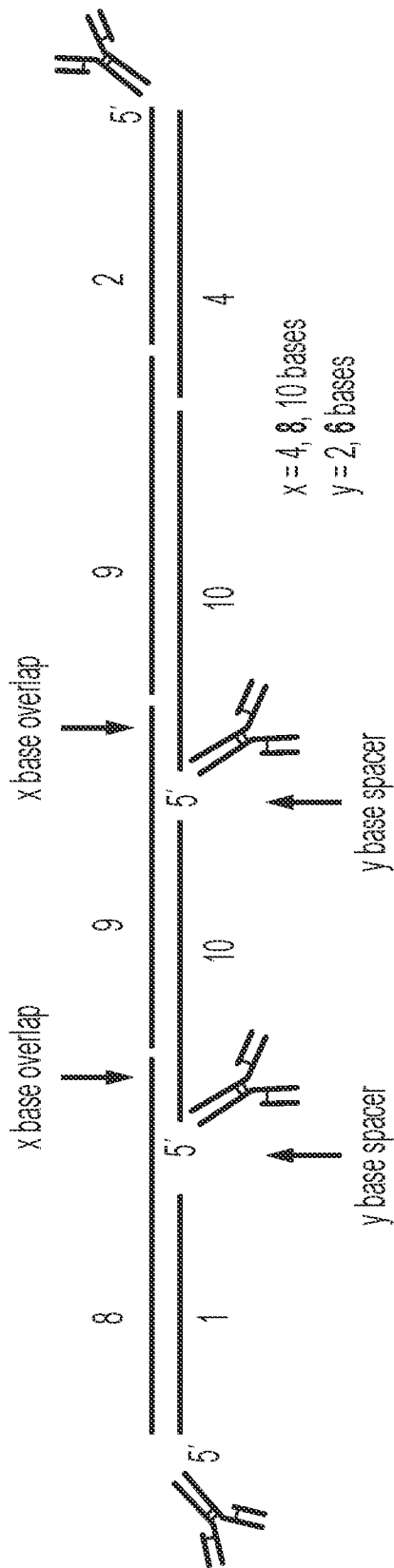


FIG. 4

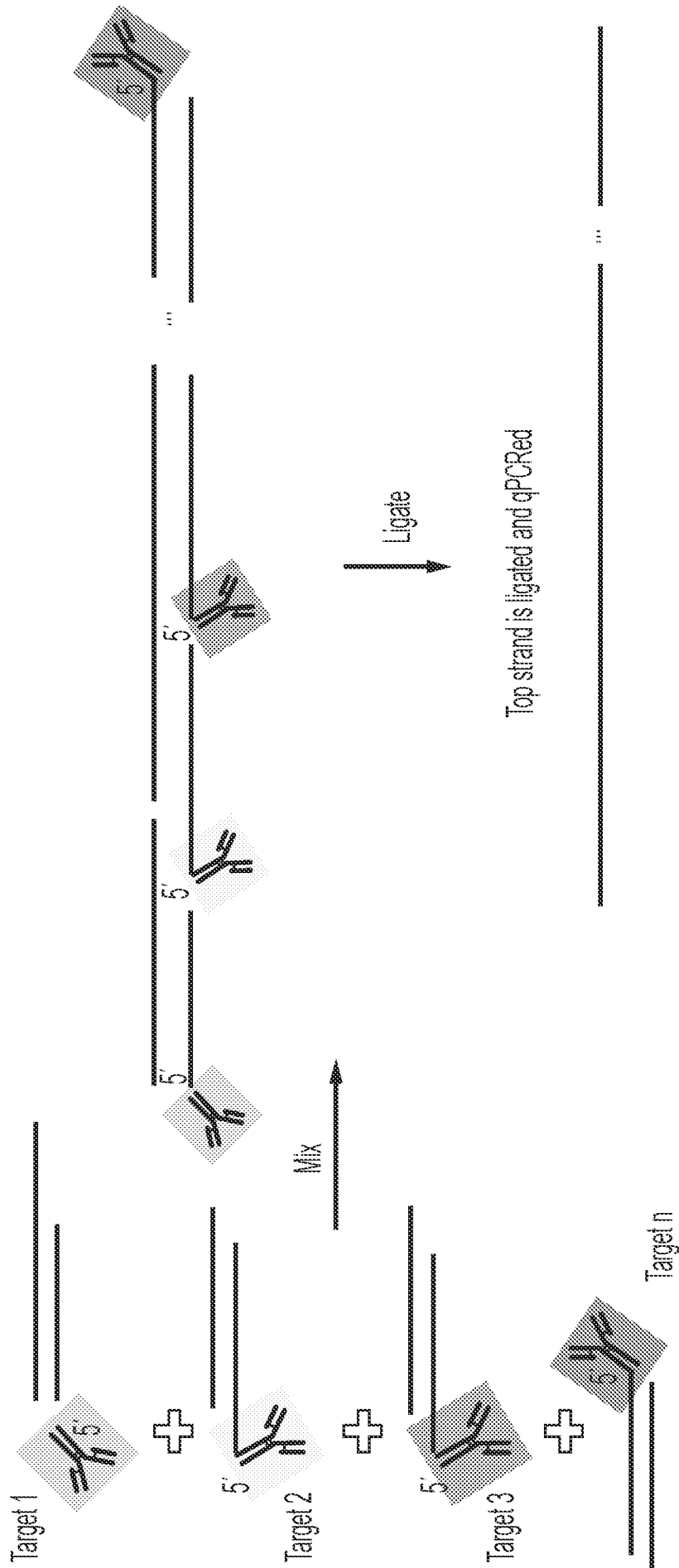


FIG. 5

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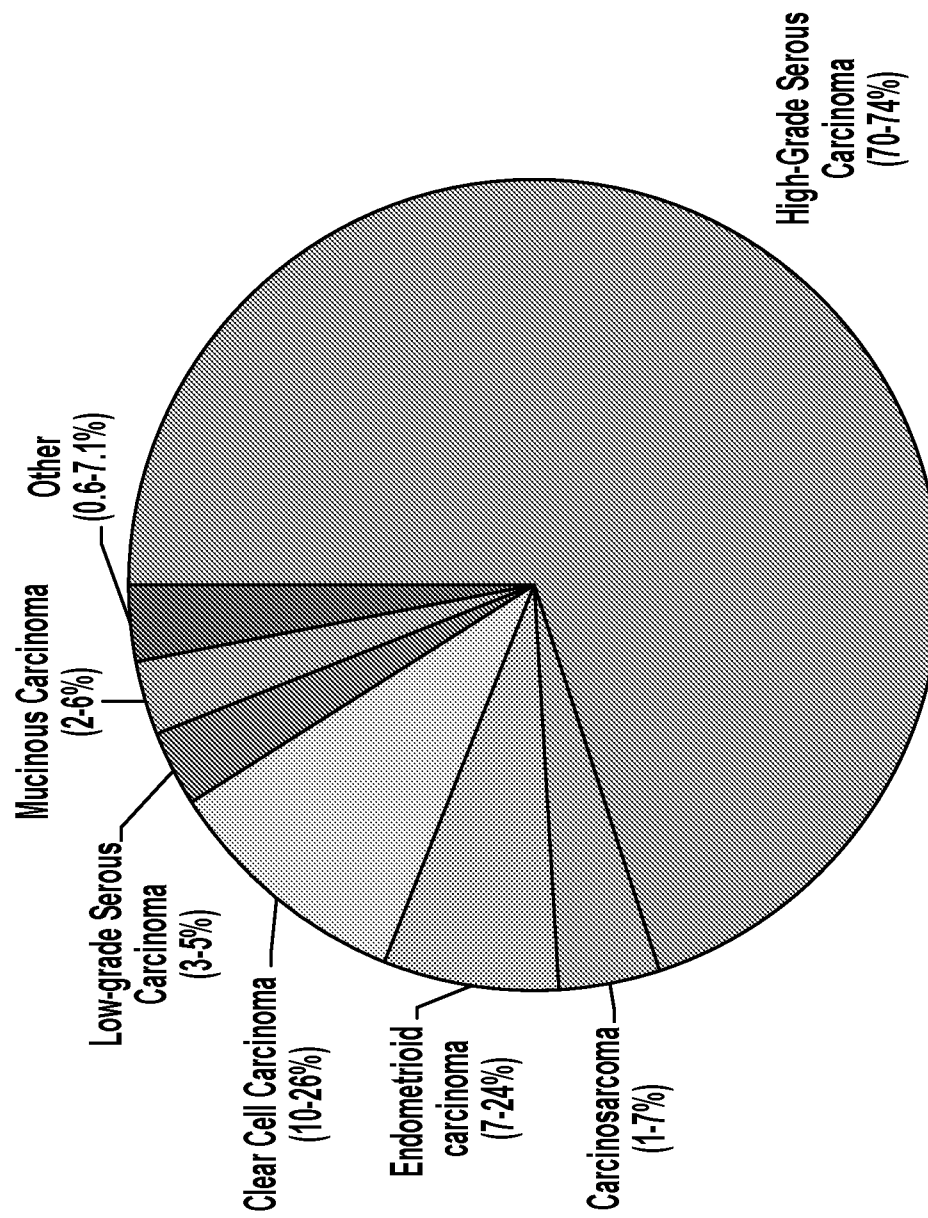


FIG. 6

Combination	Healthy Control Minimum	OC Pool 1 Delta Ct Compared to Healthy Control	OC Pool 2 Delta Ct Compared to Healthy Control	OC Pool 3 Delta Ct Compared to Healthy Control
SLC34A2, FOLR1 + FOLR1	33.4	1.6	2.6	0.5
SLC34A2, MUC16 + MUC16	29.4	-1.5	-0.7	-4.3
MUC16, FOLR1 + MUC16	32.0	4.8	7.0	1.6

FIG. 7

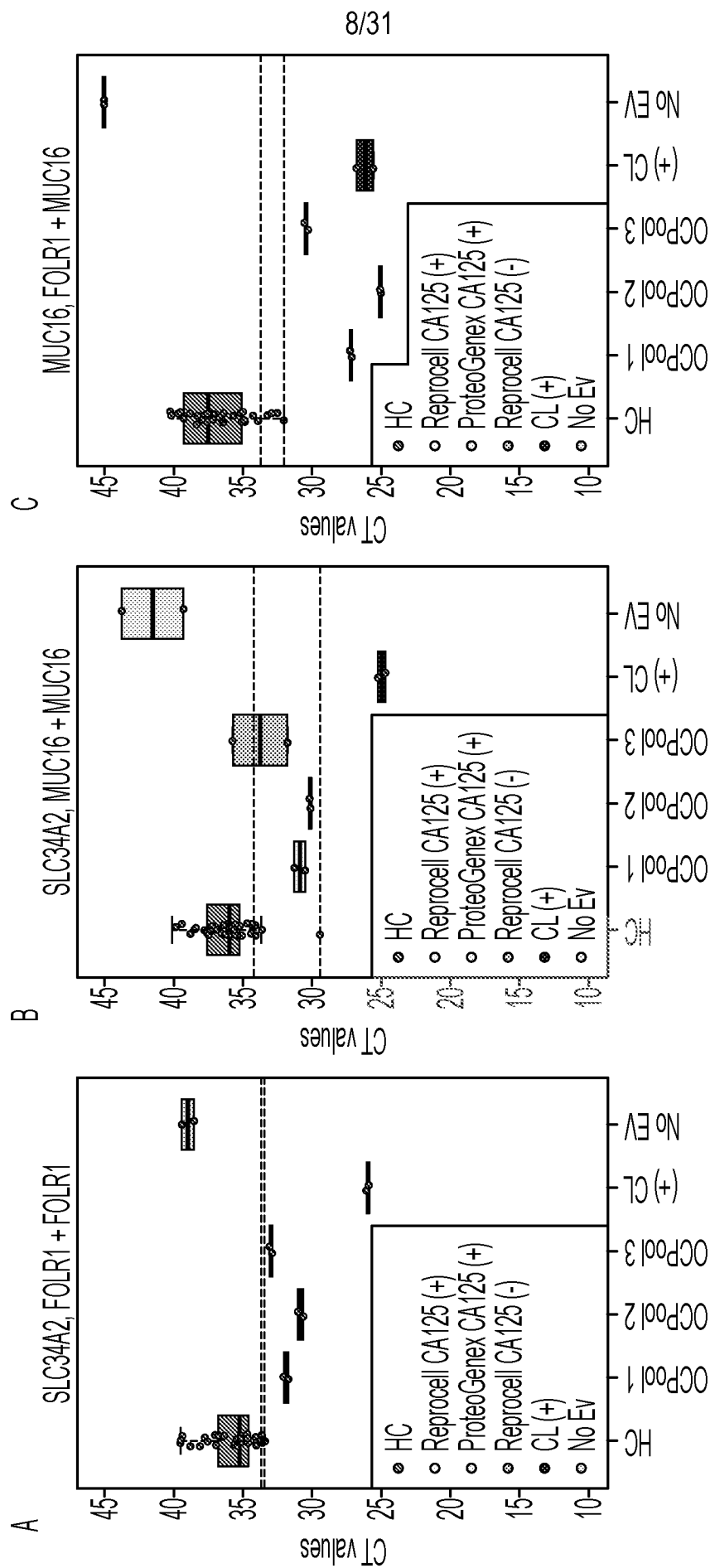


FIG. 8

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Combination	Healthy Control Minimum	OC Pool 1 Delta Ct Compared to Healthy Control	OC Pool 2 Delta Ct Compared to Healthy Control	OC Pool 3 Delta Ct Compared to Healthy Control	Delta Ct All
STn, BST2 + MUC1	31.4	12.6	11.4	3.9	9.3
MUC16, MUC16 + STn	33.5	11.2	10.8	3.0	8.4
STn, FOLR1 + MUC1	28.1	11.4	10.8	2.0	8.1
STn, FOLR1 + MUC16	29.8	10.6	10.8	2.6	8.0
STn, MSLN + MUC1	30.1	10.0	10.6	2.6	7.7
MUC16, SLC34A2 + STn	34.4	9.4	9.9	2.7	7.4
MUC16, STn + STn	25.6	9.7	10.1	2.2	7.3
STn, FOLR1 + FOLR1	28.3	10.8	10.0	0.7	7.1
MUC1, BST2 + BST2	27.8	10.3	9.5	1.1	7.0
MUC16, MUC1 + STn	31.7	8.3	9.9	2.2	6.8

FIG. 9

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Combination	Healthy Control Minimum	OC Pool 1 Delta Ct Compared to Healthy Control	OC Pool 2 Delta Ct Compared to Healthy Control	OC Pool 3 Delta Ct Compared to Healthy Control
MUC16, MUC1 + MUC16	29.8	5.3	6.7	4.5
CA19-9, SLC34A2 + SLC34A2	32.5	-0.3	3.3	3.3
T, BST2 + BST2	34.5	9.6	2.8	3.2
STn, MUC16 + MUC16 cleaved	32.1	7.4	7.7	2.8
STn, MSLN + MUC16	28.5	7.5	8.8	2.4
CA19-9, BST2 + MUC16	32.5	8.0	9.6	2.0
MUC1, BCAM + BCAM	32.2	6.9	5.2	1.7

FIG. 10

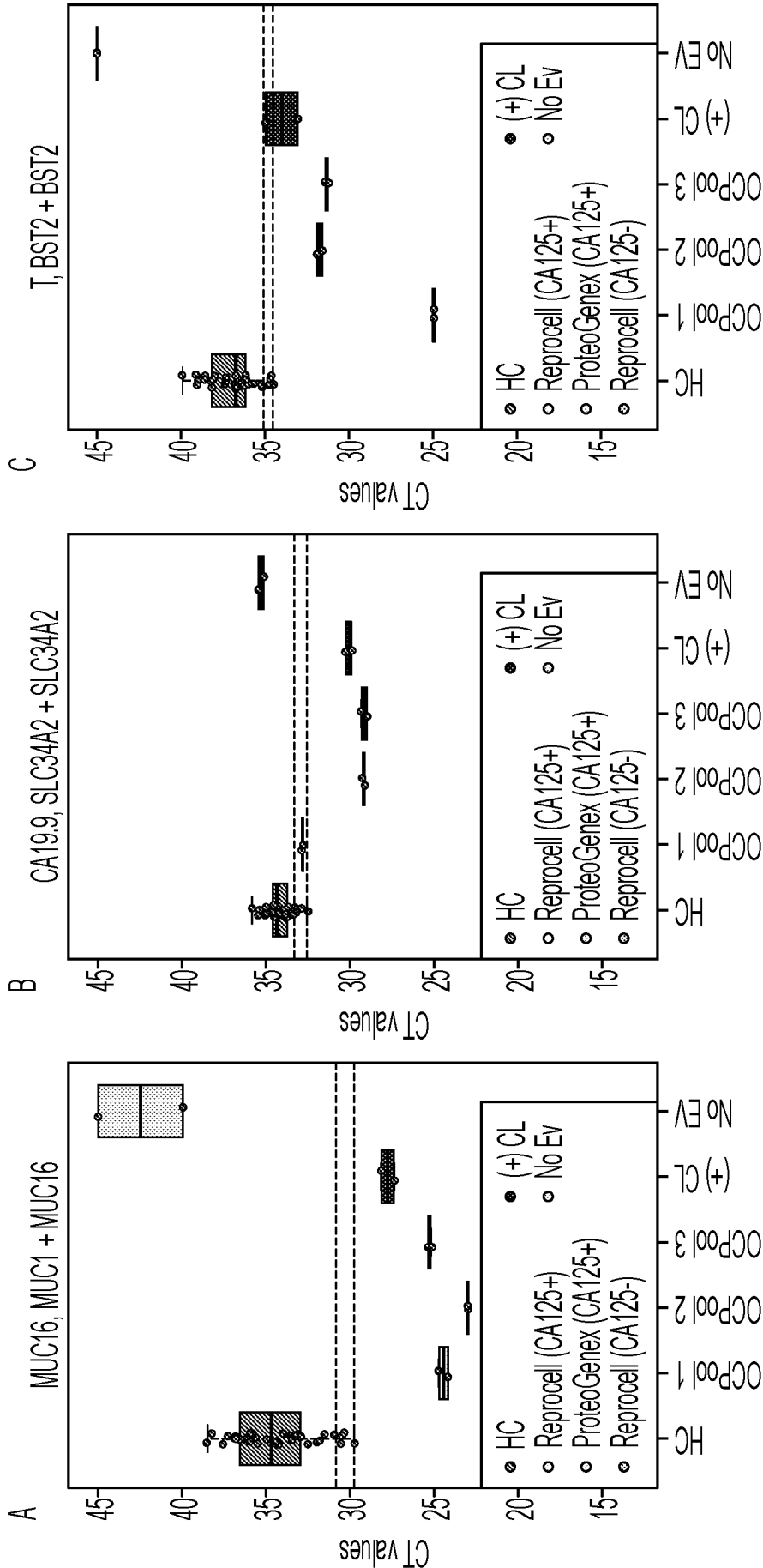


FIG. 11

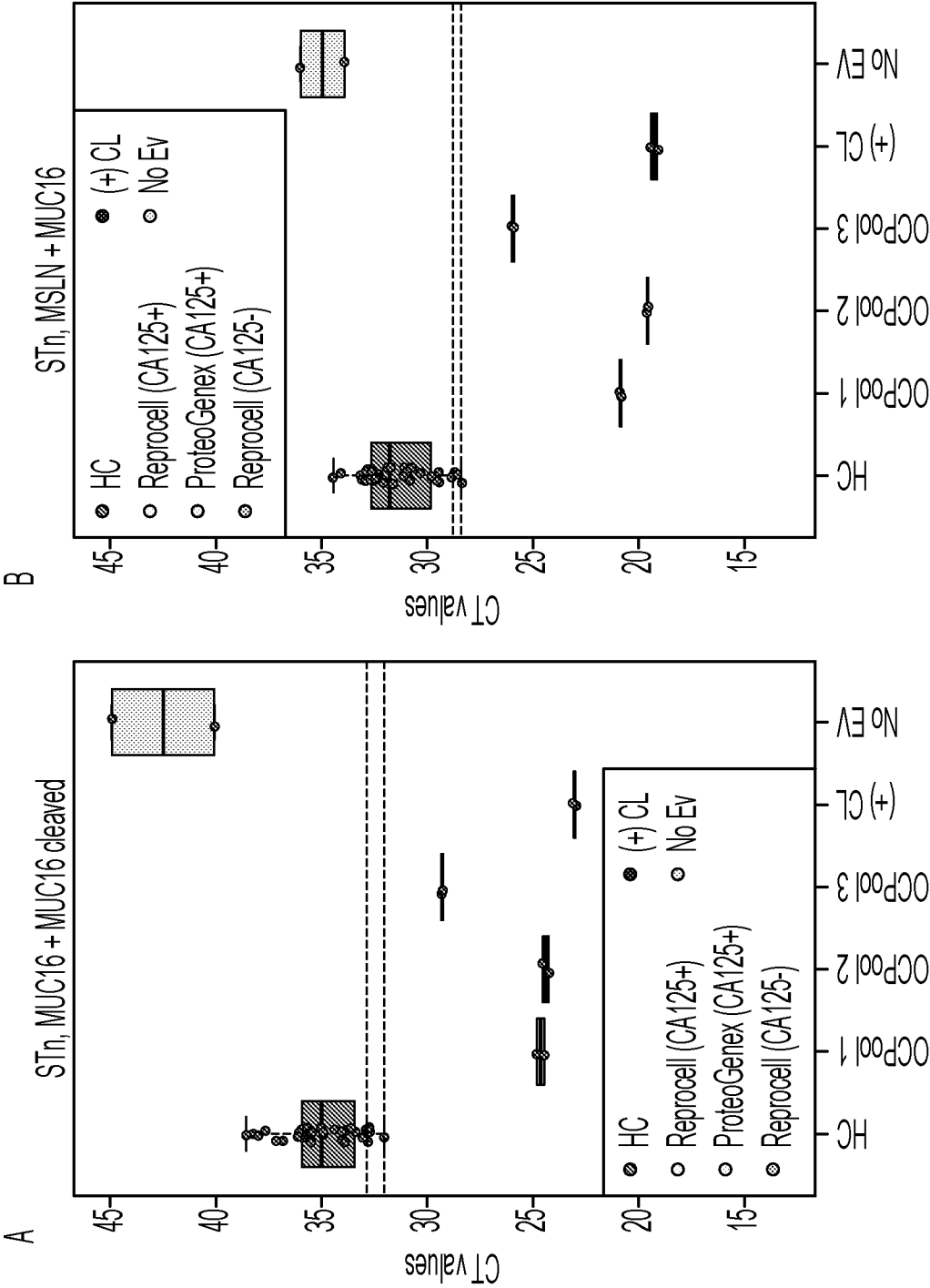


FIG. 12

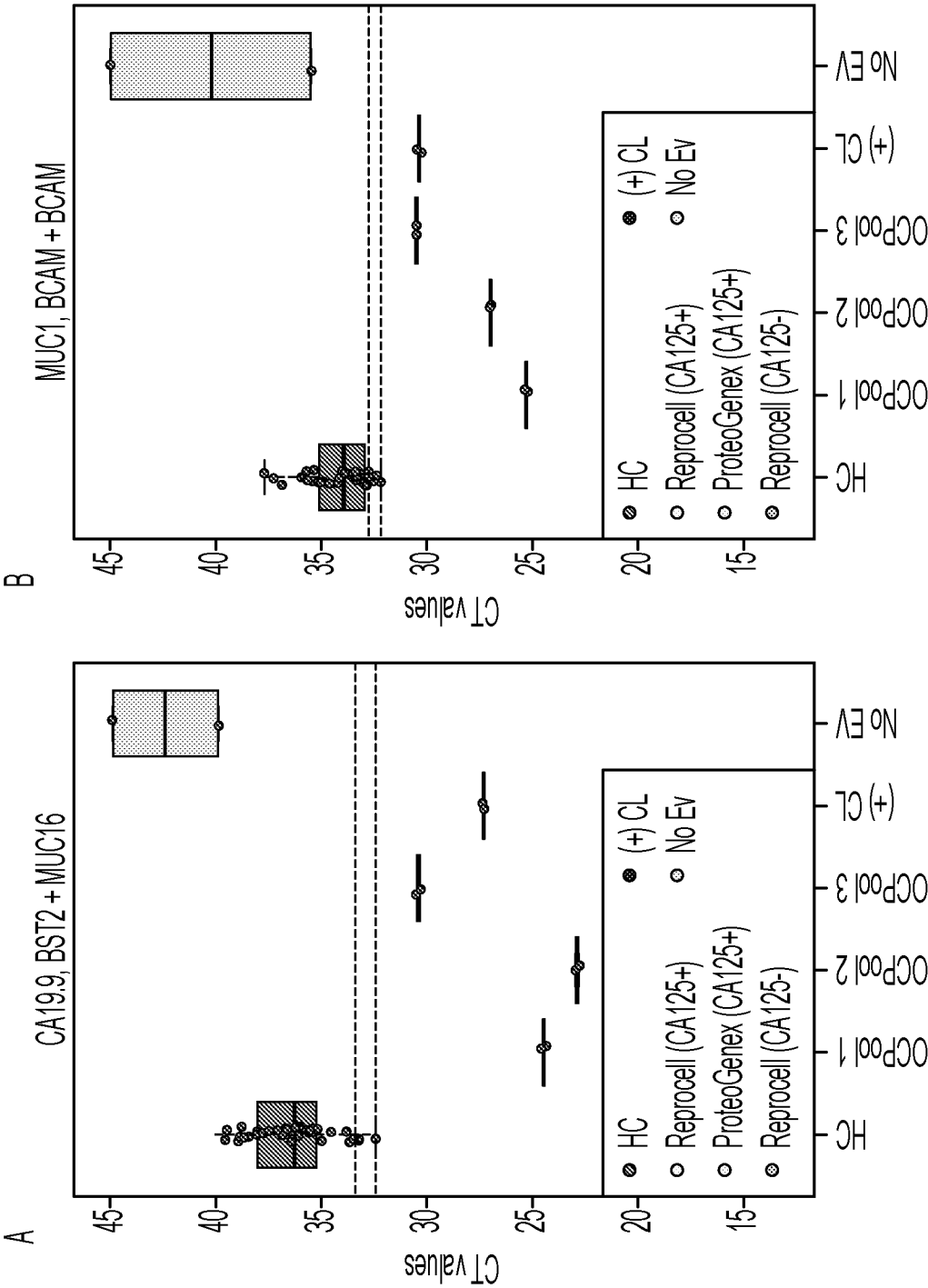


FIG. 13

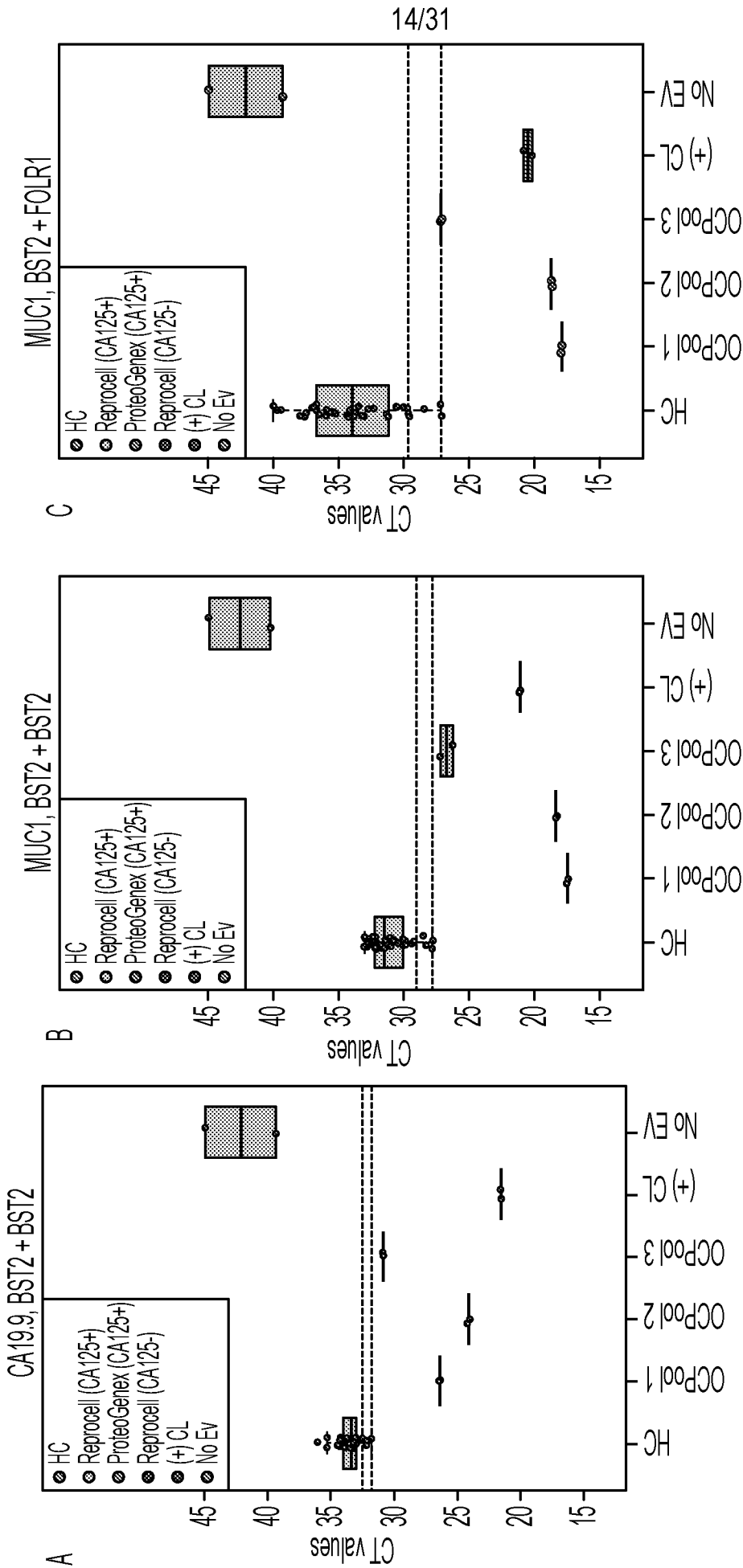


FIG. 14

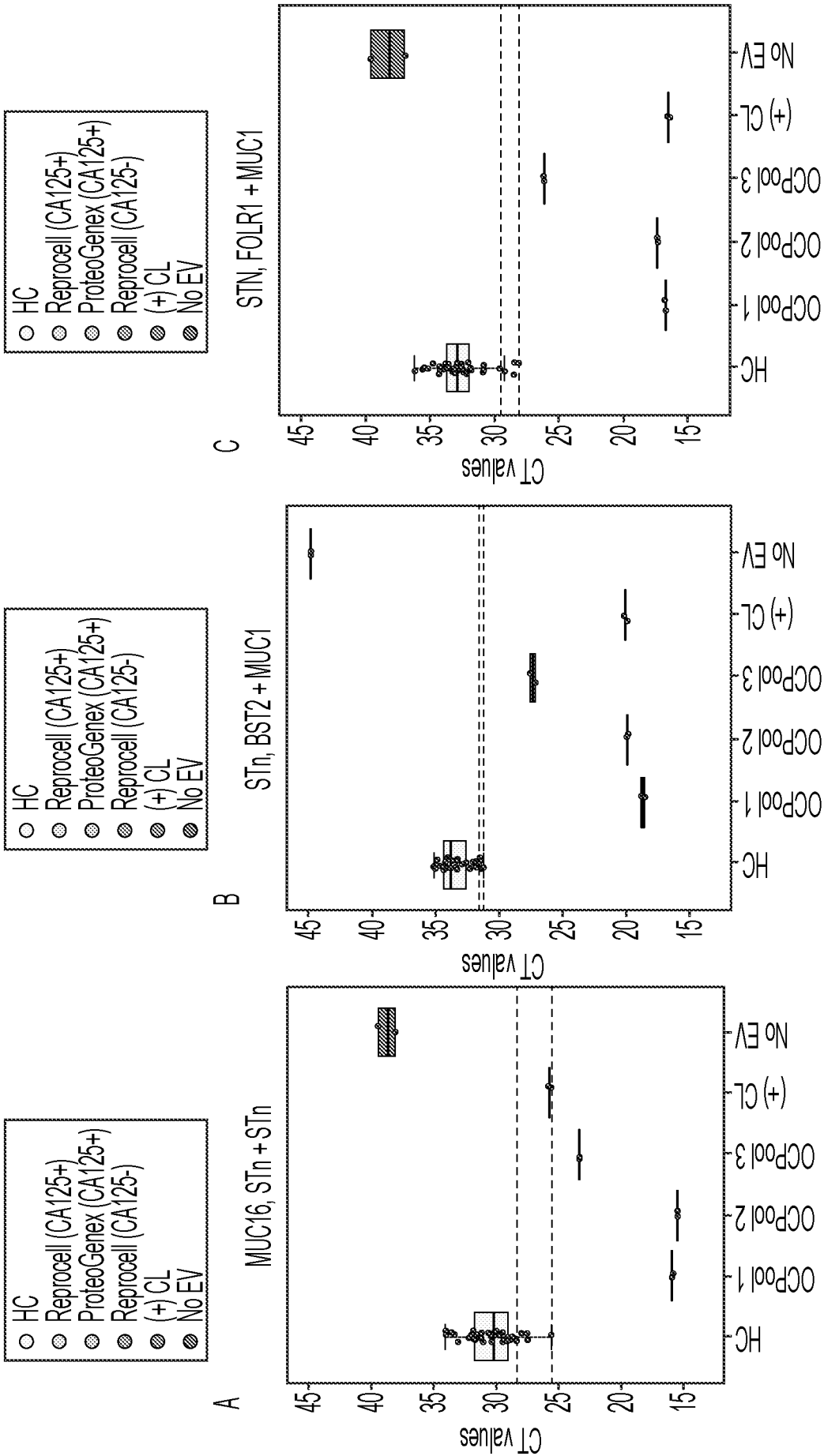


FIG. 15

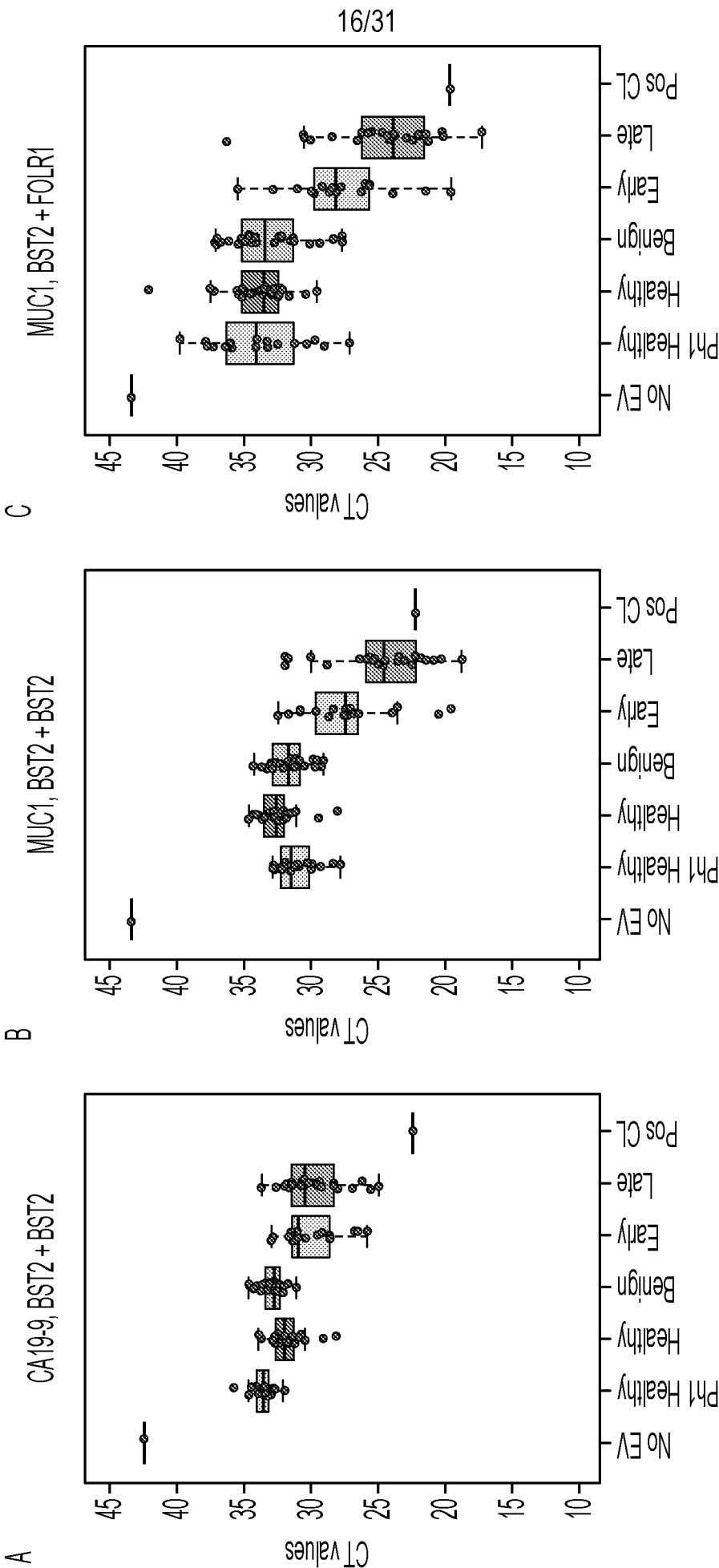


FIG. 16

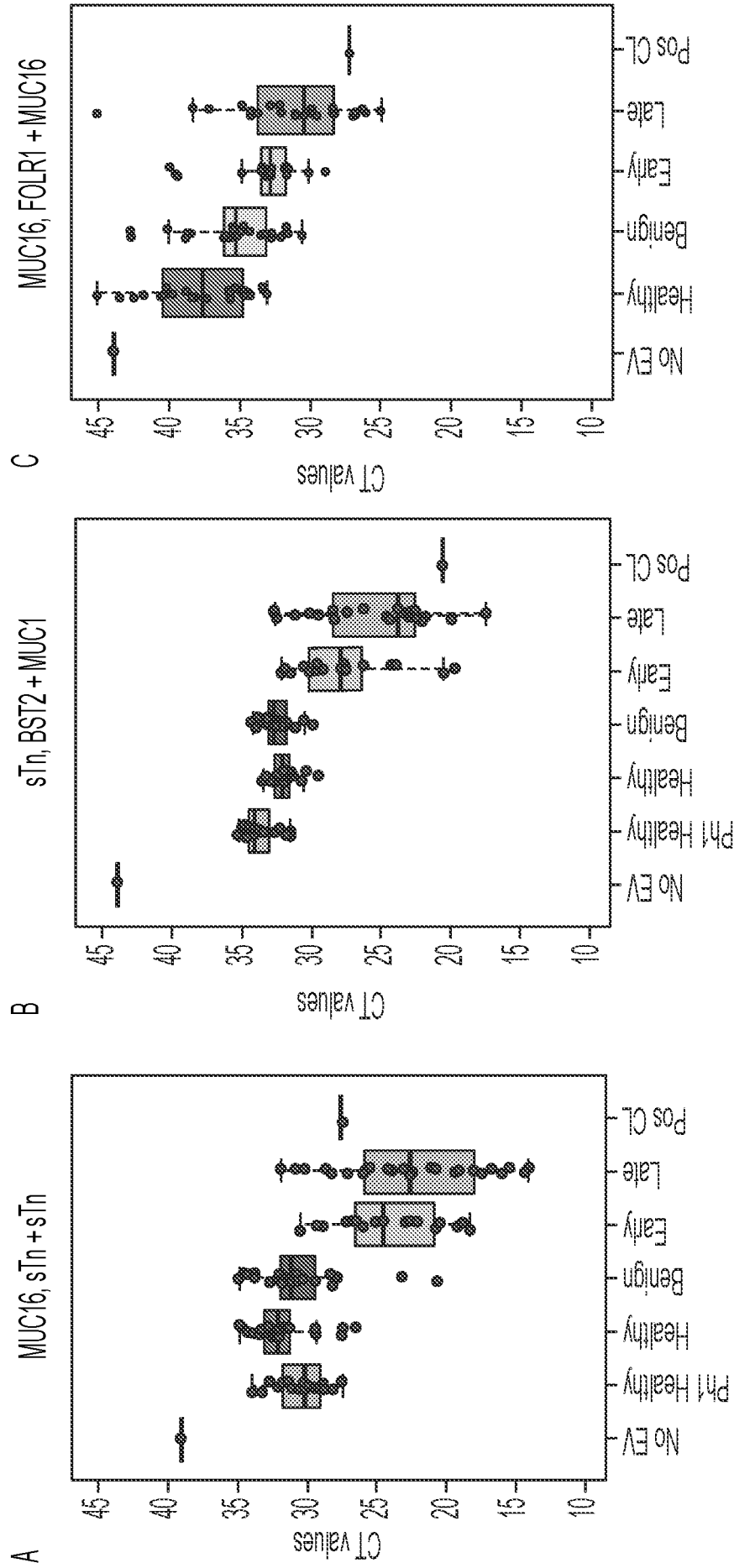


FIG. 17

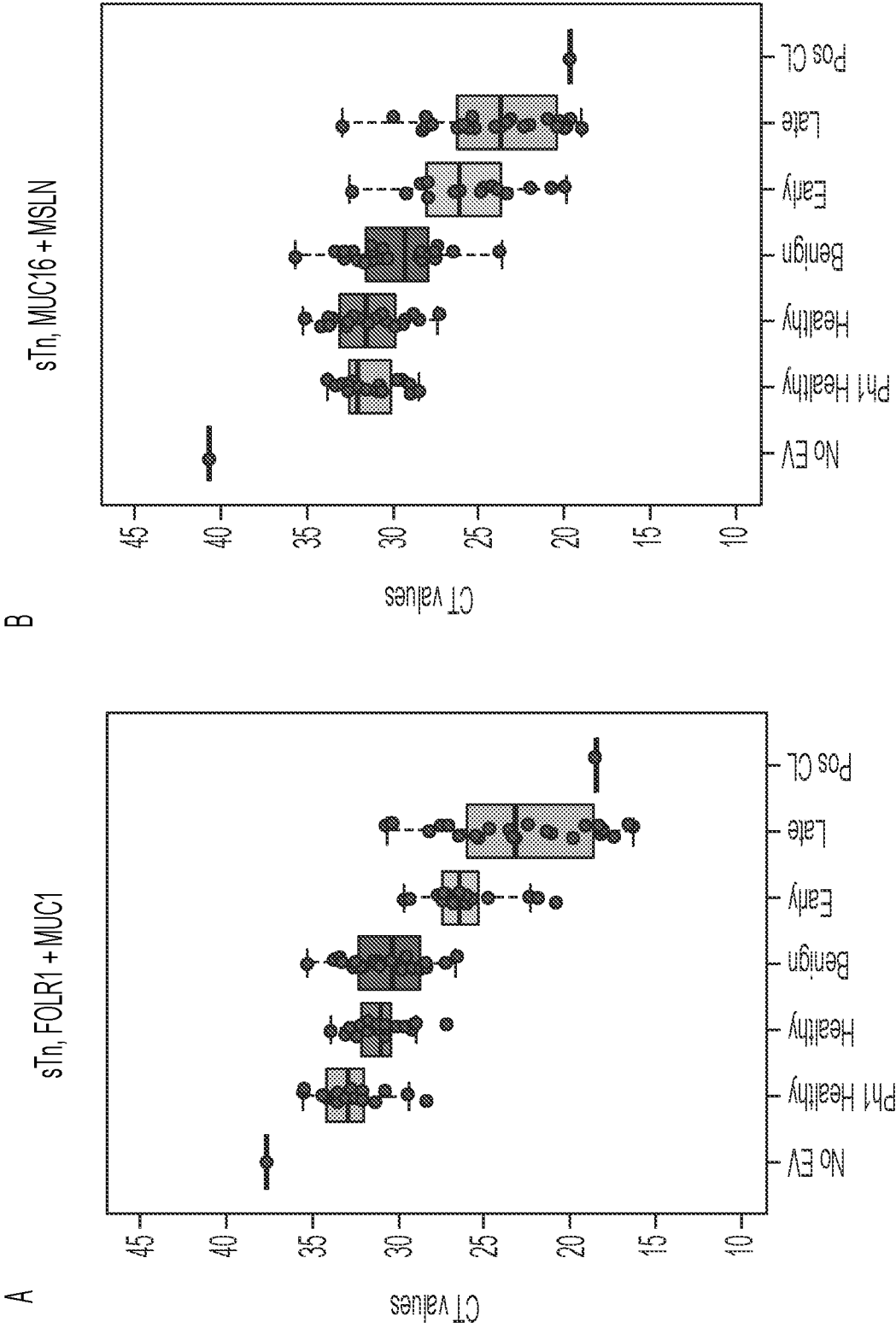


FIG. 18

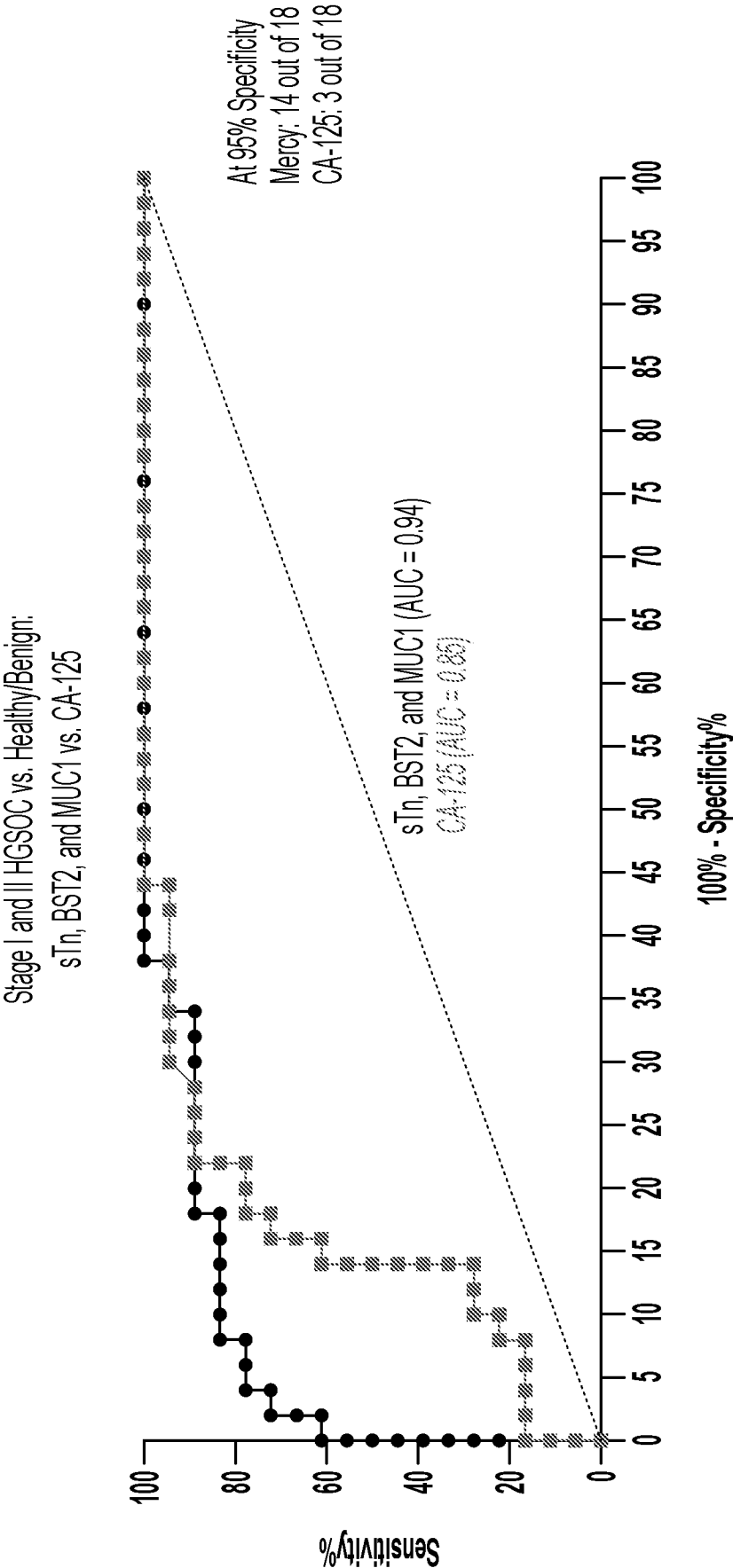


FIG. 19

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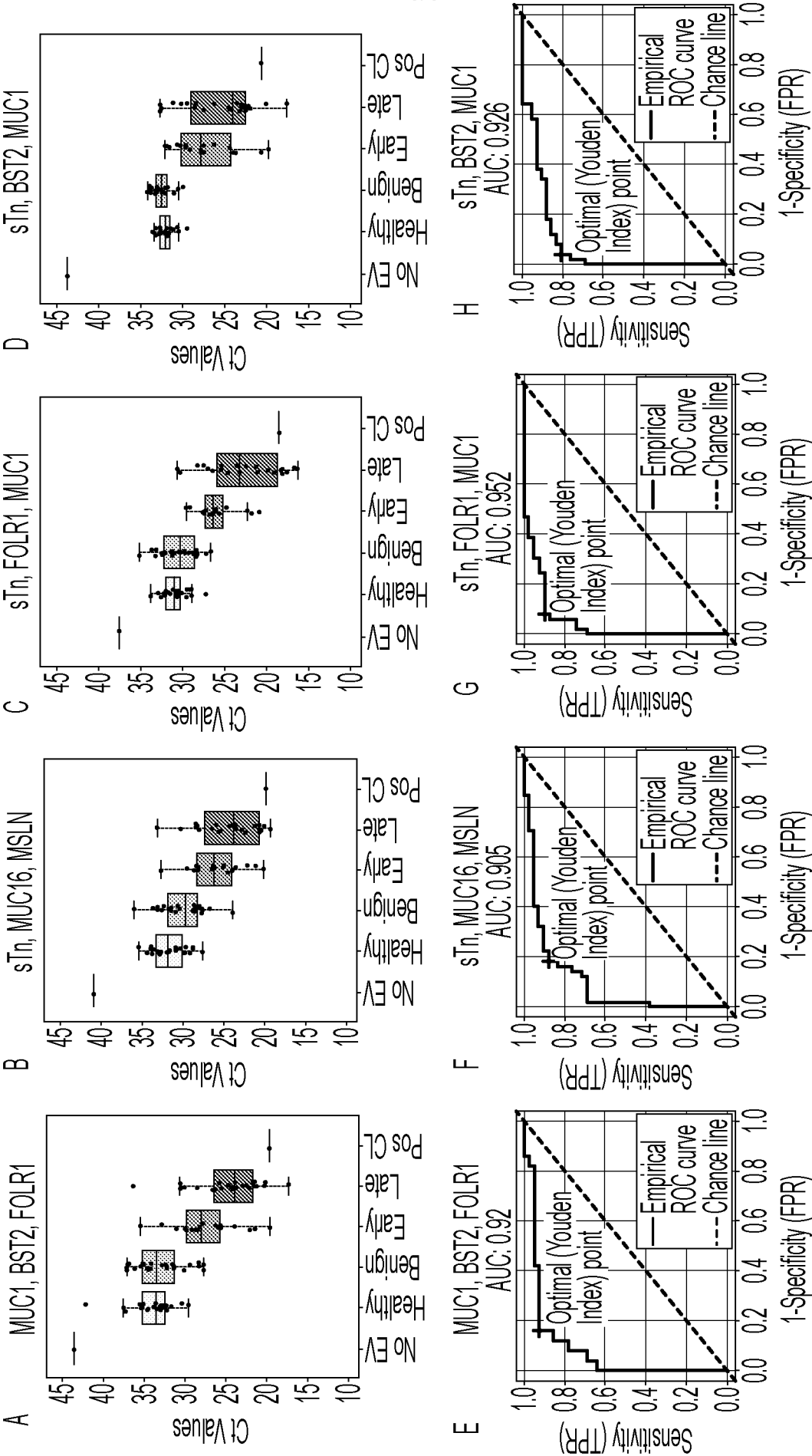


FIG. 20

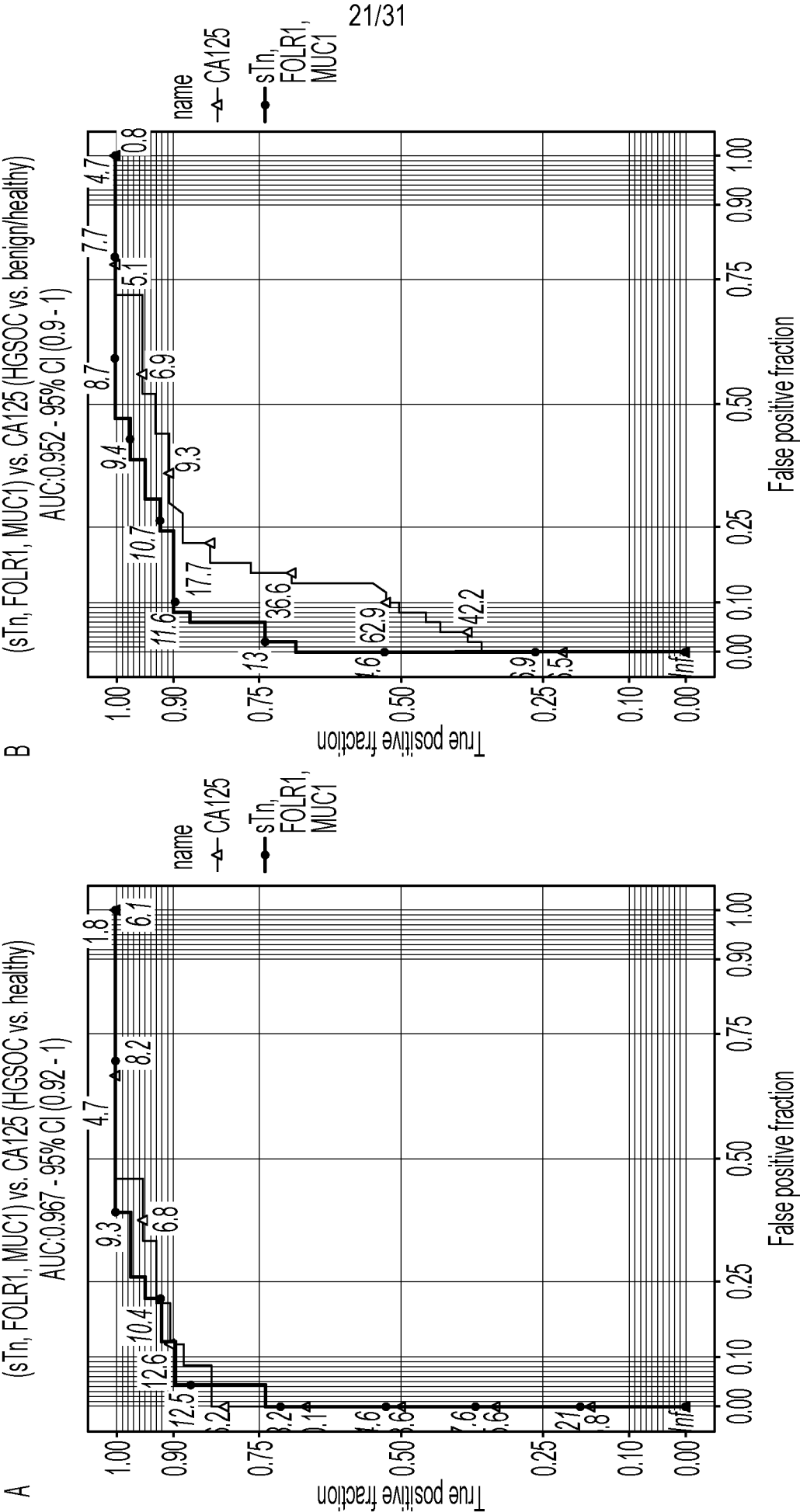


FIG. 21

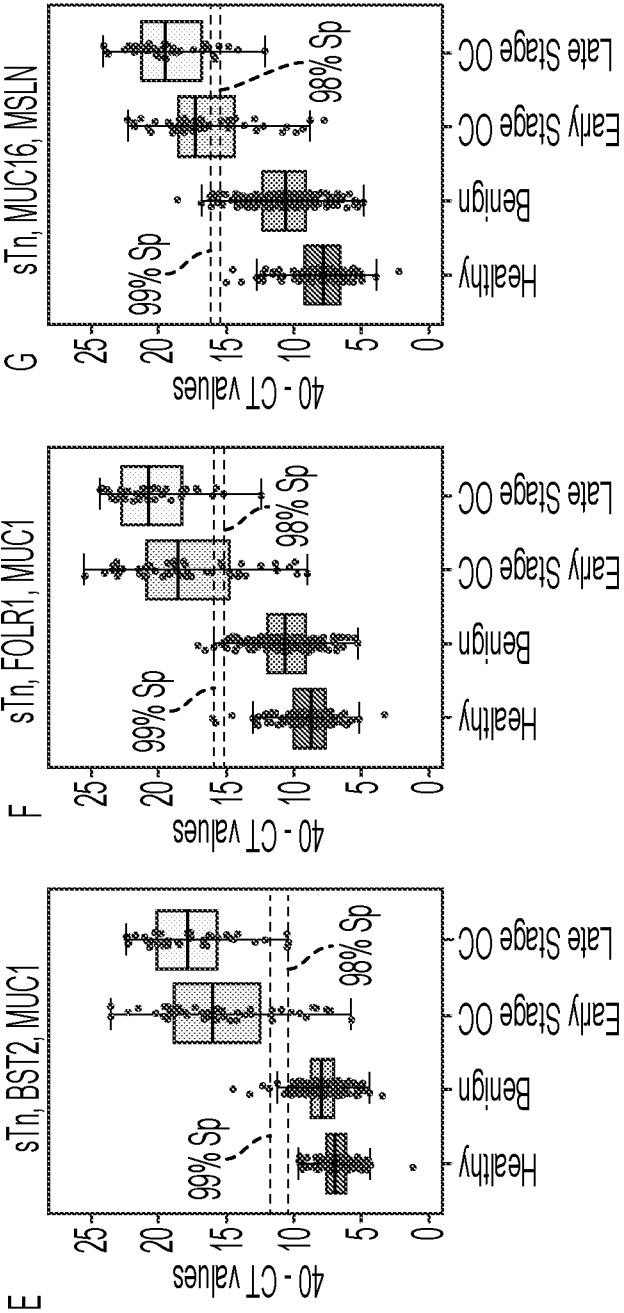
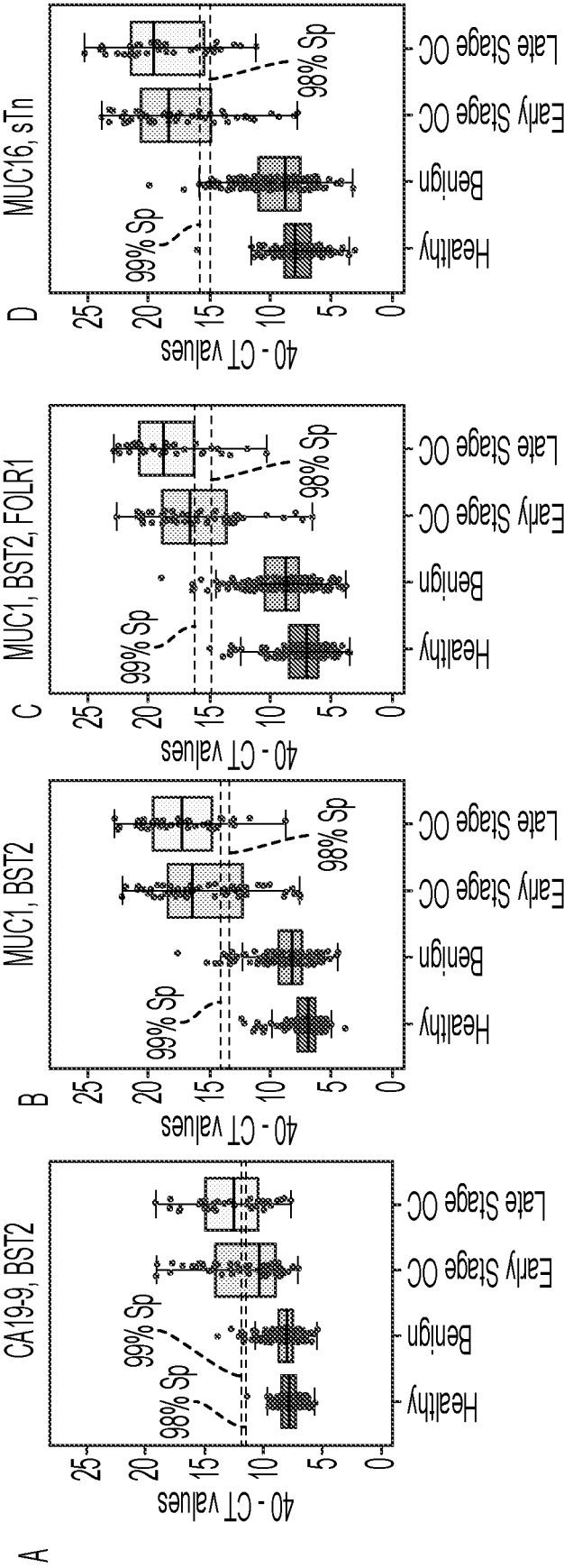


FIG. 22

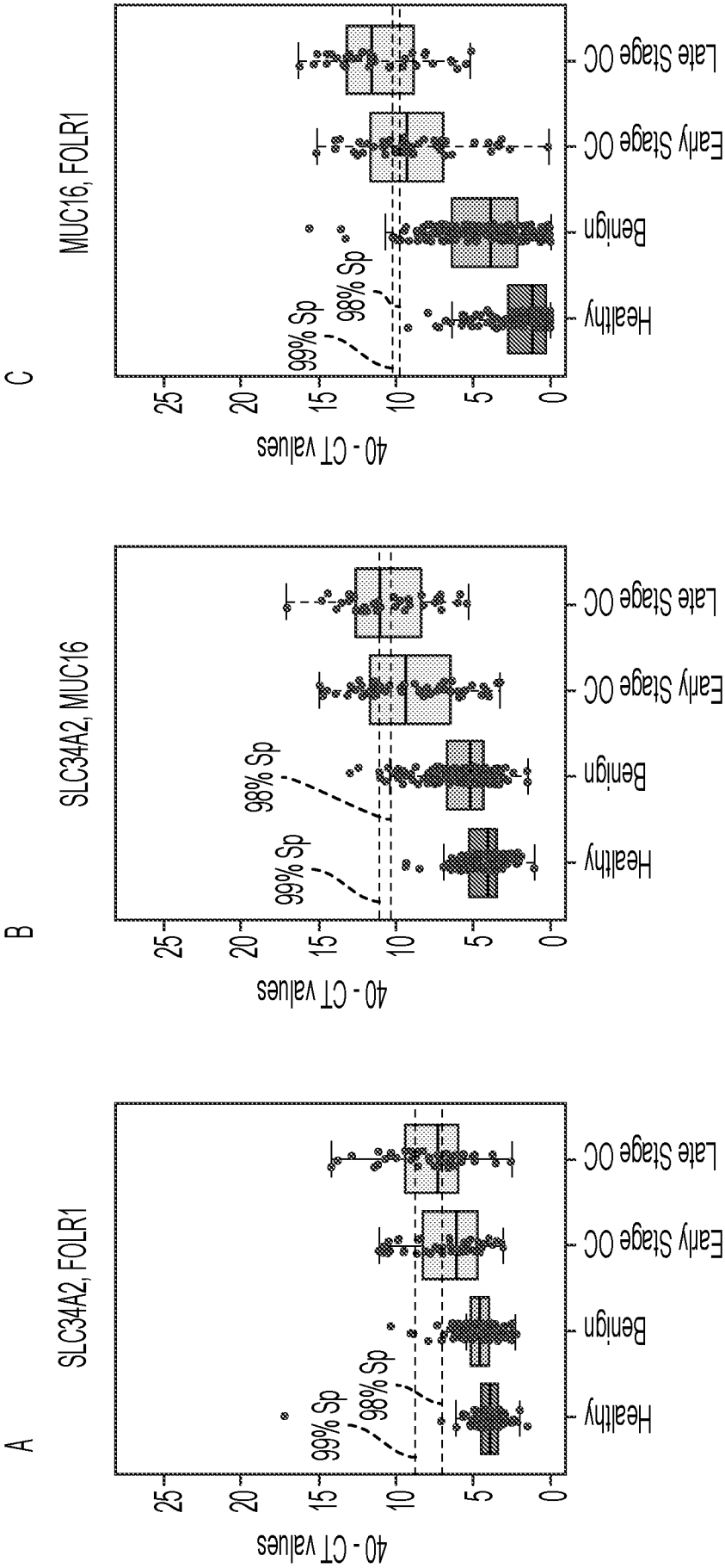


FIG. 23

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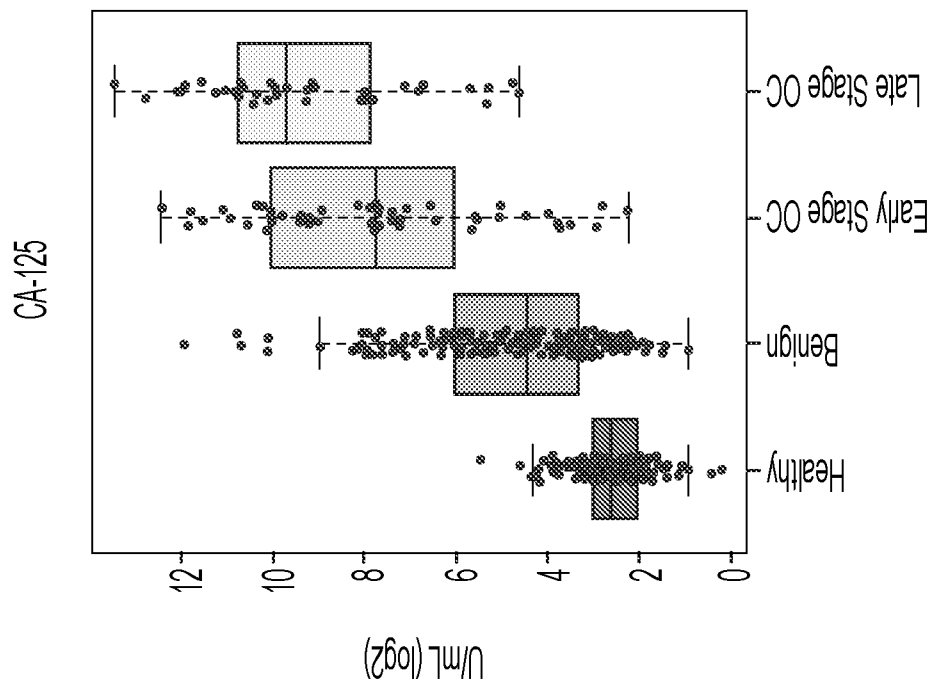


FIG. 24

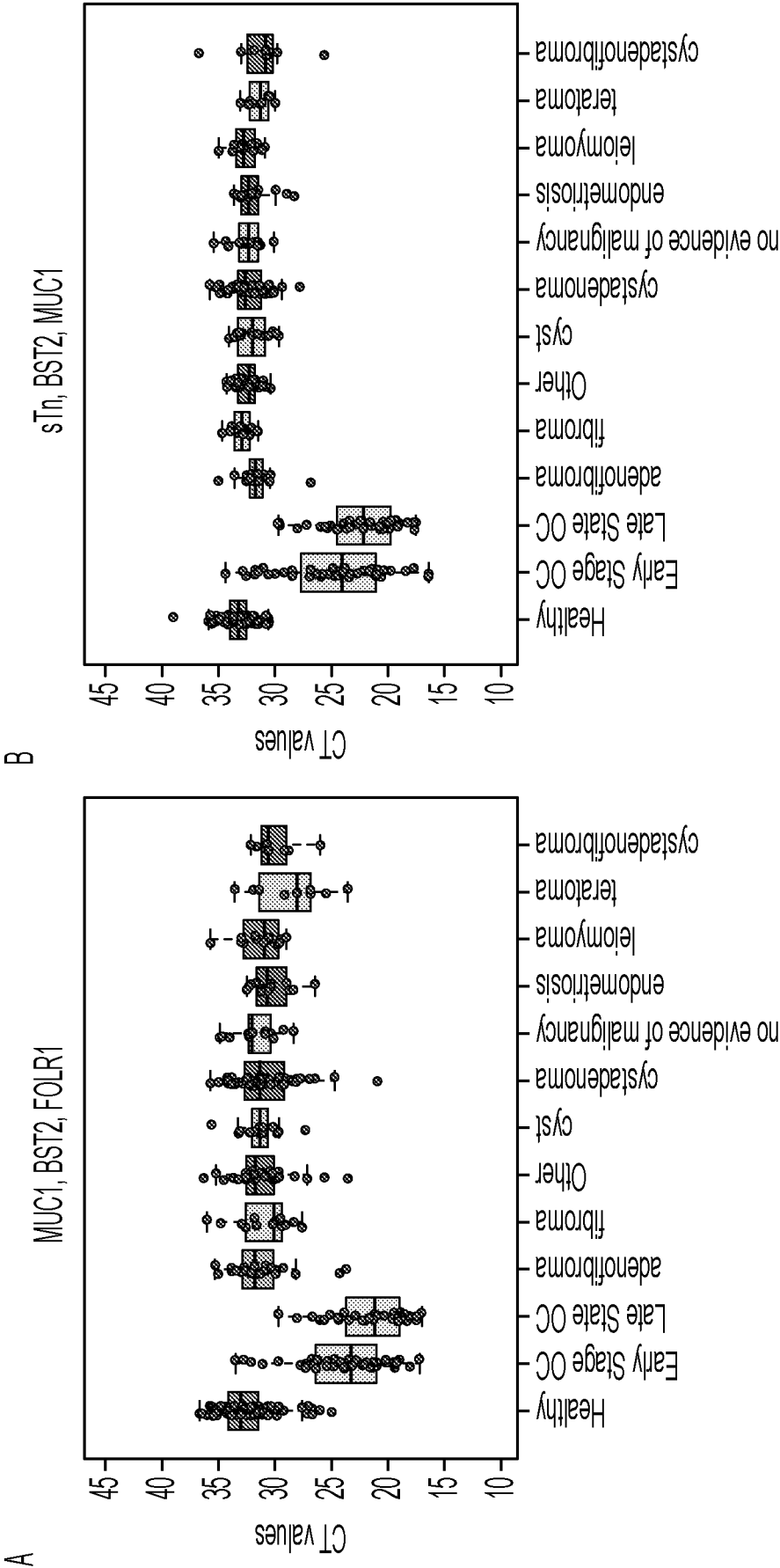
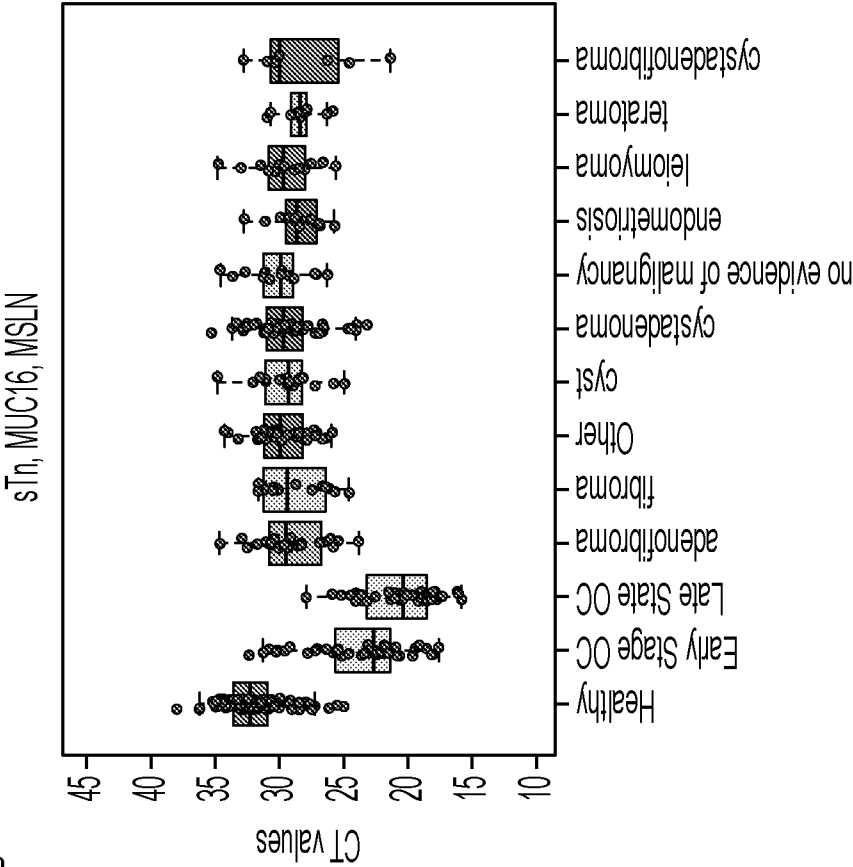


FIG. 25

D



C

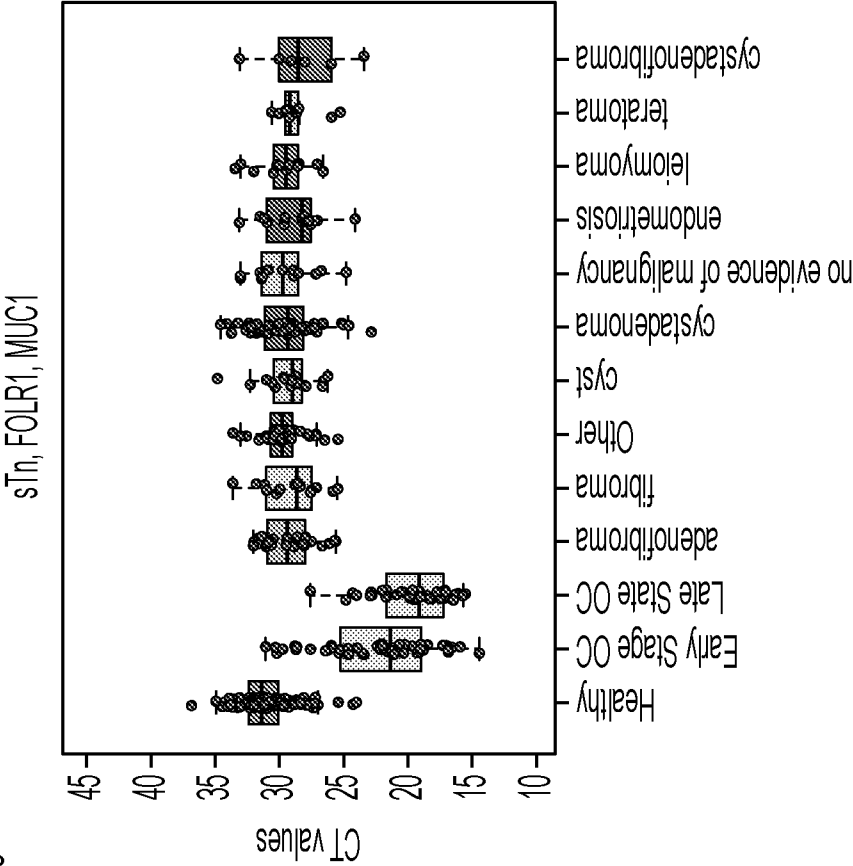


FIG. 25
CONTINUED

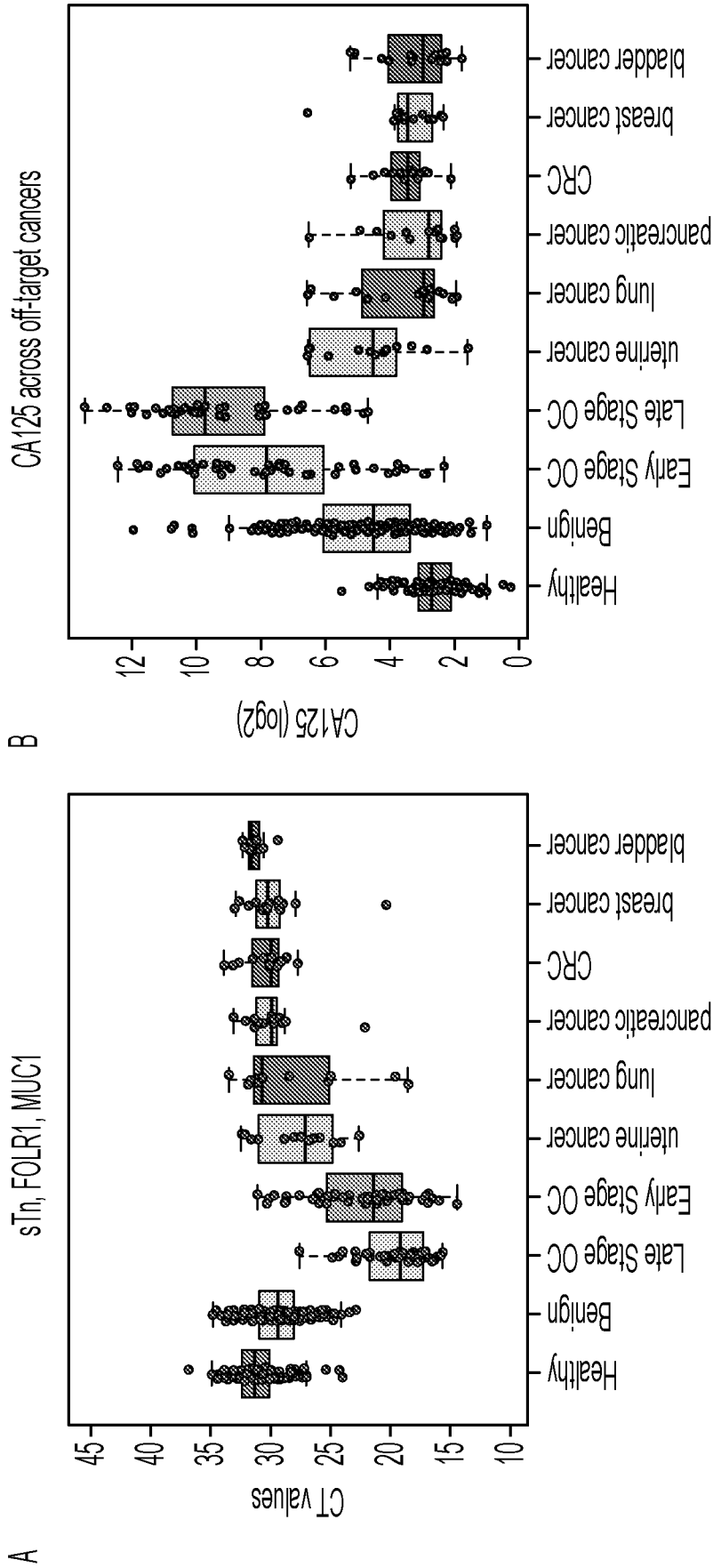


FIG. 26

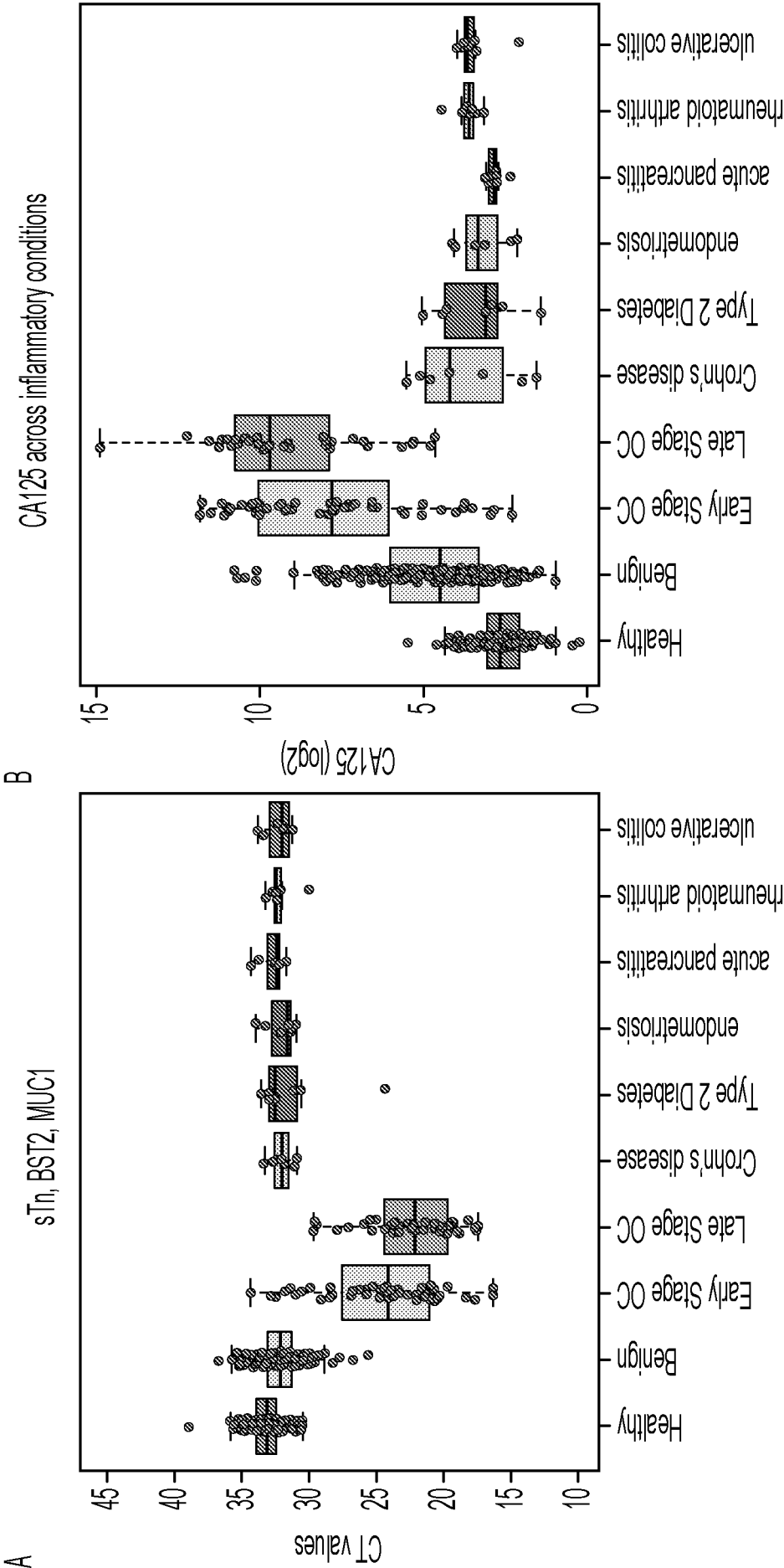


FIG. 27

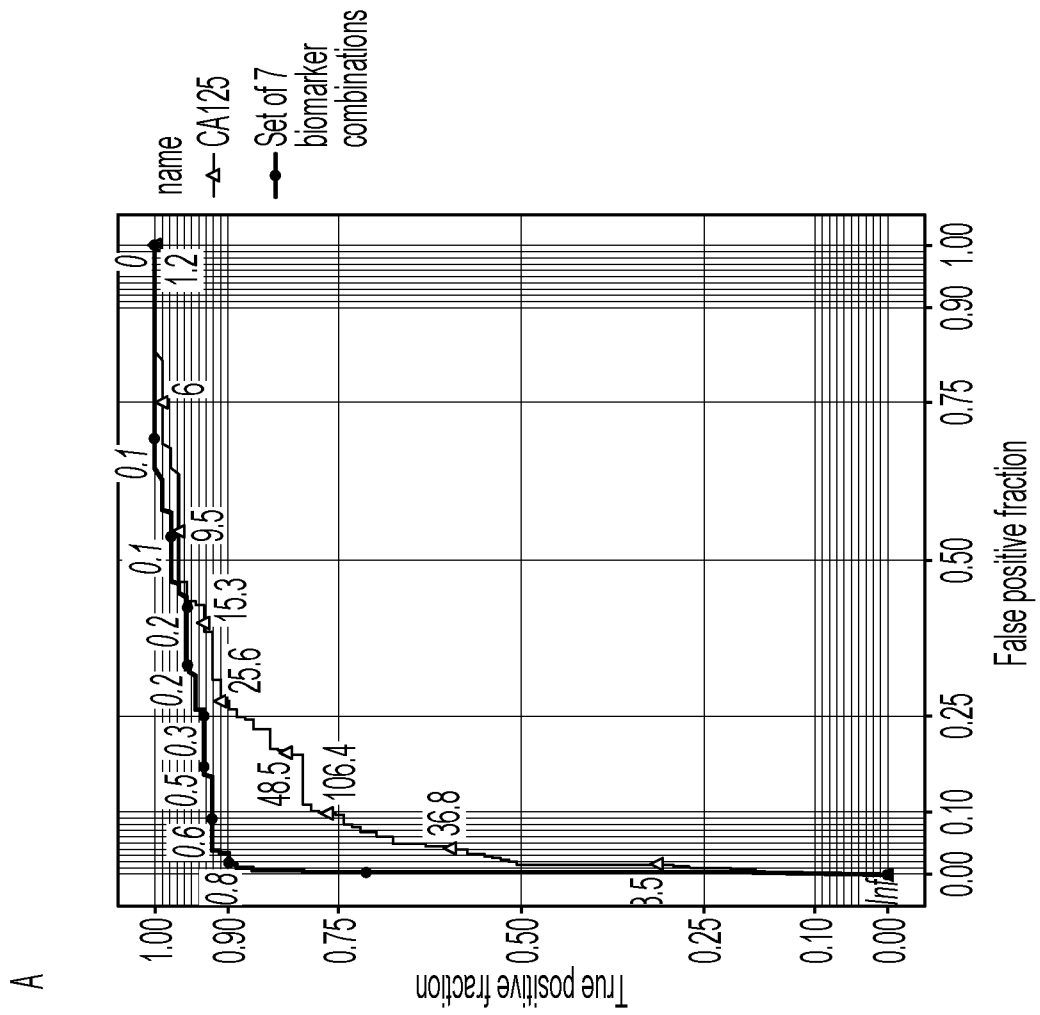


FIG. 28

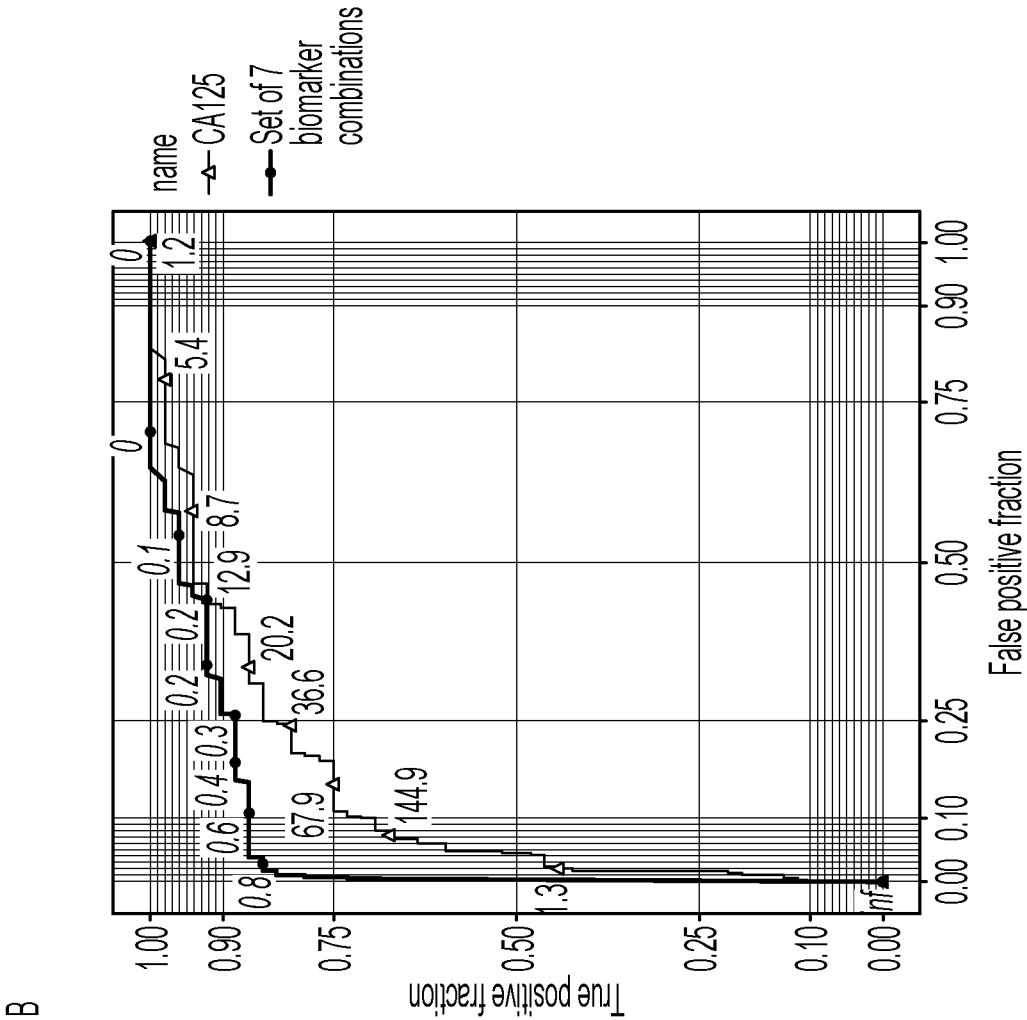
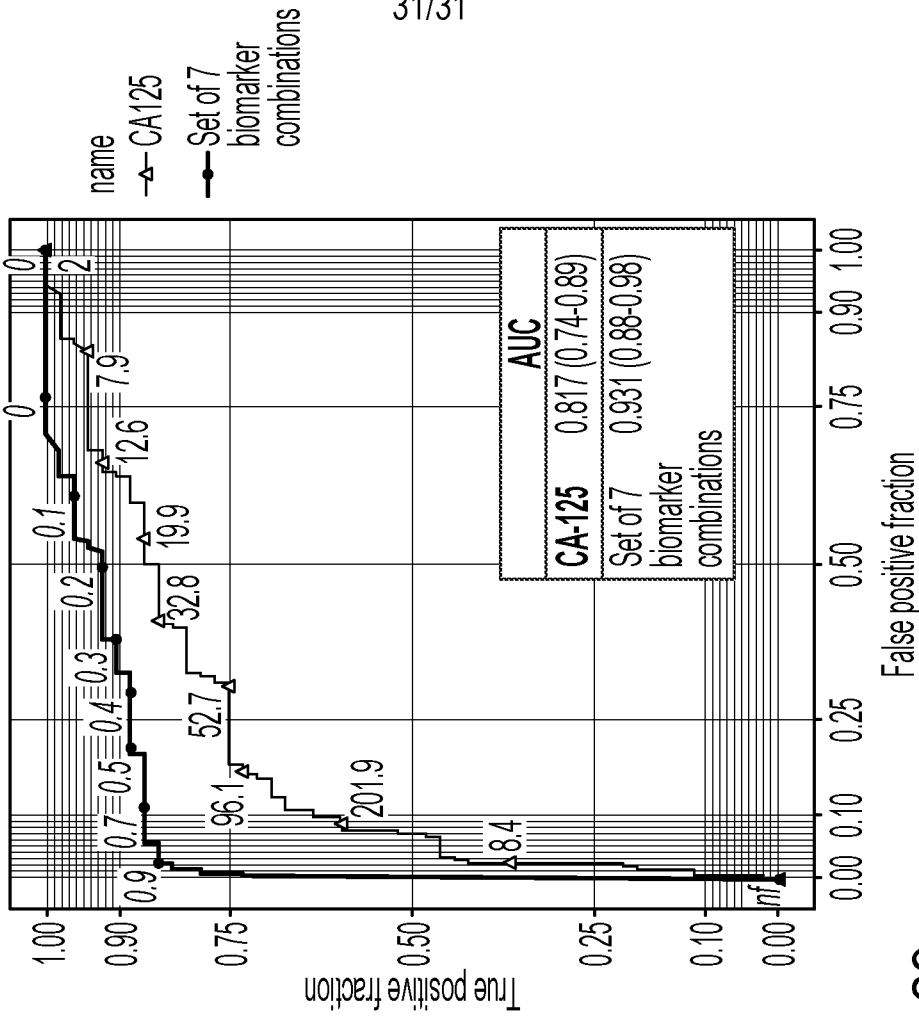


FIG. 28
CONTINUED

B



A

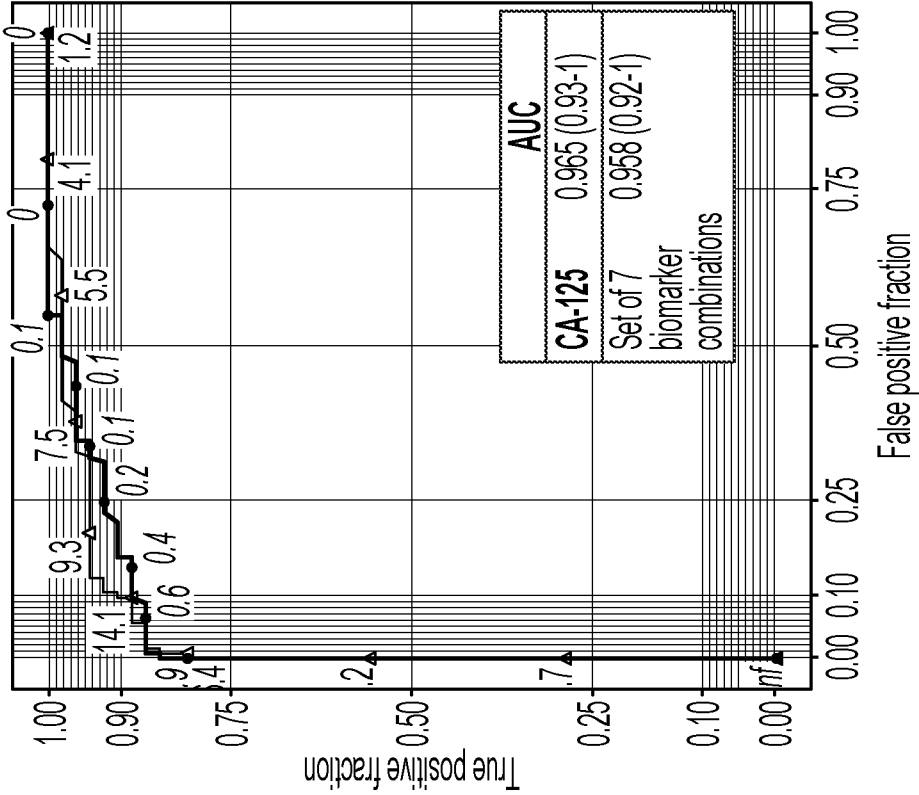
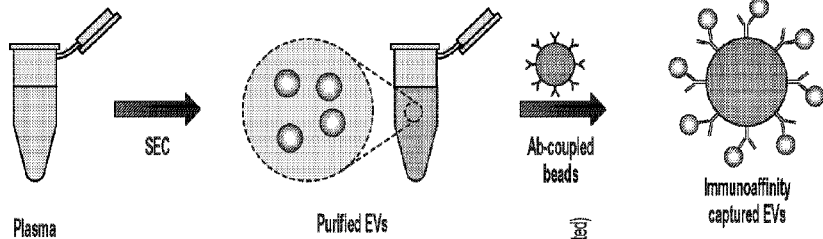


FIG. 29

1A



1B

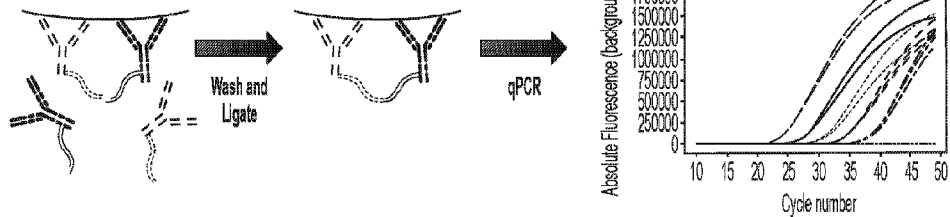


FIG. 1