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DESCRIPTION

FIELD

[0001] This disclosure relates to systems, kits, and methods using oligonucleotide probes, for detection of nucleic acid target sequences (e.g., genomic DNA or RNA), for gene copy number enumeration, and/or for tissue diagnostics.

BACKGROUND

[0002] Probes have been developed for a variety of diagnostic and research purposes. Hybridization of chromosome or gene-specific probes has made possible detection of chromosomal abnormalities associated with numerous diseases and syndromes, including constitutive genetic anomalies (such as microdeletion syndromes, chromosome translocations, gene amplification and aneuploidy syndromes), neoplastic diseases, as well as pathogen infections. Detection of genetic changes in these regions can provide diagnostic and prognostic information for patients and in some cases, inform treatment decisions.

[0003] Dual detection and enumeration of human chromosome 17 (CHR17) and human epidermal growth factor receptor 2 (HER2) is important for the selection of appropriate patients for HER2 targeted therapy in breast cancer (Wolff AC, et al., J Clin Oncol 2007, 25:118-145; Gruver AM, et al., J Clin Pathol 2010 Mar; 63(3):210-9), but existing probes that may be used for such dual detection and enumeration are known for requiring long assay times to obtain specific and sensitive detection.

[0004] Double-stranded CHR17 centromere probes are typically generated from the p17H8 plasmid sequence, which is directed to human CHR17's alpha satellite. The alpha satellite of human CHR17 contains a ~2,700 base pair higher order repeat unit that consists of 16 monomers and is present in 500 to 1,000 copies per CHR17 (Waye JS, et al., Molecular and Cellular Biology, Sept. 1986, p. 3156-3165). Double-stranded HER2 probes are typically generated from bacterial artificial chromosomes (BACs) and span the HER2 gene (Dal Lago L, et al., Mol Cancer Ther 2006, 5:2572-2579; Gruver AM, et al., J Clin Pathol 2010 Mar; 63(3):210-9). These double-stranded probes have repetitive sequences that are common to centromere regions of other human chromosomes. Consequently, a significant drawback to these probes is the noise-generating repetitive elements. That is, probes to the centromere regions typically have significant cross-reactivity to other chromosome centromeres. As such, blocking DNA has been required to be used in conjunction with these probes to reduce non-specific binding (*See* Pinkel and Gray, U.S. Patent No. 5,447,841). Assays employing these probes require extensive hybridization time to achieve sufficient hybridization because of their double-stranded nature and the required competition with the blocking DNA, *e.g.,* about 6 to 18 hours. This time consuming step reflects low hybridization efficiency, in part due to self-hybridization of the double-stranded probe and in part because of the competition with the blocking DNA. Libraries of BAC probes are also cumbersome to generate and maintain, laborious to purify, and are prone to contamination. The benchmark and ground-breaking

assay using this technology was disclosed by Nitta *et al.* in 2008 and is commercially available as the INFORM HER2 Dual ISH DNA Probe Cocktail, Ventana Medical Systems, Catalog Number: 780-4422 (Nitta et al. Diagnostic Pathology, 3:41, 2008).

[0005] Recently, Matthiesen and Hansen (Matthiesen SH, et al., PLoS One, 2012; 7(7), 2012) claimed that with no change in the HER2 and CHR17 probe configuration, substitution of ethylene carbonate (EC) for formamide in the hybridization buffer reduced FISH hybridization time and requires no blocking DNA. The HER2 IQFISH pharmDx™ assay (Dako) was introduced to the market based on this technology. While a useful technique, fluorescence *in situ* hybridization (FISH) has its drawbacks. Implementation of conventional FISH requires a dedicated fluorescence imaging system and well-trained personnel with specific expertise, making this system incompatible with some clinical workflows. Furthermore, when compared to bright-field *in situ* hybridization (ISH) approaches, FISH studies provide relatively limited morphological assessment of overall histology, lack stability of the fluorescent detection signal(s) over time, and have a higher overall cost of testing.

[0006] In an effort to alleviate drawbacks associated with clone-based probes, investigators have proposed the use of "specific primers" to generate probes from genomic DNA (Navin et al., Bioinformatics 22:2437-2438 (2006)). However, this process is cumbersome and time consuming in that it requires multiple specific amplification reactions and downstream processing with upfront hands-on time (See also Yamada et al., Cytogenet Genome Res. 1-7 (2010)).

[0007] For some applications, the use of single-stranded probes has a distinct advantage over the use of double-stranded probes. For example, single-stranded probes generally have higher sensitivity than double-stranded probes because a proportion of the denatured double-stranded probe renatures to form probe homoduplexes, thus preventing their capture of genomic targets in the test samples (Taneja K et al., Anal Biochem, 166, 389-398 (1987), Lewis ME, et al., Peptides, 6 Suppl 2:75-87 (1985); Strachan T, Read AP, Human Molecular Genetics. 2nd edition. New York: Wiley-Liss (1999); Kourilsky P, et al., Biochimie, 56(9):1215-21 (1974)). Several laboratories have reported that single-stranded probes provide higher hybridization sensitivity than double-stranded probes (An SF, et al., Mol Cell Probes, 6(3):193-200 (1992); Hannon K, et al., Anal Biochem, 212(2):421-7 (1993); Cox KH, et al., Dev Biol., 101(2):485-502 (1984)).

[0008] Synthetic single-stranded oligonucleotide probes have been used to detect genomic targets, mostly for FISH. For example, Bergstrom et al., Designing Custom Oligo FISH Probes for the Detection of Chromosomal Rearrangements in FFPE Tissues, American Society of Human Genetics 2013 Meeting (2013) reported SureFISH probes comprising thousands of unique, long single-stranded oligonucleotides with fluorescence labels. The oligonucleotide sequences tile across the targeted chromosomal region of translocation breakpoints for the detection of chromosomal rearrangements. Although Bergstrom discloses single-stranded probes, the probes were not directed to CHR17 and the Bergstrom reference does not appear to provide any solutions to the difficulties associated with CHR17 probes, such as specificity and robustness to detect CHR17 polymorphisms in a human population. Also, the Bergstrom reference does not disclose assays (and probes) for gene copy number enumeration wherein a target probe and a reference probe are used in combination to calculate a target gene to reference chromosome ratio.

[0009] The use of single-stranded oligonucleotide probes for genomic targets has been extremely limited. For example, U.S. Pat. No. 8,445,206 (Bergmann et al., 2012) describes a set of at least 100 single-stranded oligonucleotide probes directed against (or complementary to) portions of the HER2 gene. The disclosure appears to be limited to detection of the HER2 gene target without a reference probe (*e.g.,* CHR17), which is useful for gene copy number assessment as the HER2/CHR17 ratio is diagnostically important as evidenced from the teachings of Wolff AC, et al., J Clin Oncol 2007, 25:118-145.

[0010] Comparative genome hybridization (CGH) assays may be used for providing information on the relative copy number of one sample (such as a tumor sample) compared to another (such as a reference sample, for example a non-tumor cell or tissue sample). Thus, CGH may be used for determining whether genomic DNA copy number of a target nucleic acid is increased or decreased as compared to the reference sample. However, CGH does not provide information as to the exact number of copies of a particular genomic DNA or chromosomal region.

[0011] For genomic labeling of CHR17, a previous 42-mer oligonucleotide derived from p17H8 was demonstrated to be specific to CHR17. But, because of significant differences in the sizes of the 42-mer CHR17 probe and the preferred oligomeric HER2 probes (ranging from about 100 bp to about 400 bp) disclosed herein, the dual HER2- CHR17 ISH assay required a lengthy procedure to sequentially detect HER2 and CHR17 signals under different stringency wash temperatures (72 °C for HER2 and 59 °C for CHR17). Importantly, dual ISH experiments using the 42-mer CHR17 probe and single-stranded HER2 probes of a similar size did not resolve the incompatibility of the probe sets (*See* FIG. 14A-D and Example 2). Further, even if the incompatibility between the 42-mer CHR17 probe and the single stranded HER2 probes were resolved, a single oligonucleotide probe (*e.g.,* the 42-mer CHR17 probe) specific for only a one monomer of the alpha satellite's 16 monomers as taught by Nitta would not be sufficient to detect CHR17 throughout the human population since each individual human being may carry different combinations of the monomers and their related variants (Waye JS and Willard HF, NAC 1986; 14(17); Willard, H.F. et al, 1987, Genomics, 1; Warburton, P.E. and Willard, H.F., 1995, J. Mol. Evol., 41). Thus, the 42-mer CHR17 probe as taught by Nitta would not be robust enough across the entire population.

[0012] Despite the appeal of the use of a single-stranded CHR17 probe, workers in this field thought it is not possible to make short, single-stranded CHR17 probes that are specific enough to CHR17 (*e.g.*, specific enough to eliminate the need for blocking DNA), and robust enough to sufficiently detect CHR17 throughout the human population. One of the reasons for this understanding is that it was believed that the fundamental repetitive nature of alpha satellite DNA makes the likelihood of finding short oligonucleotides specific enough to CHR17 impossibly improbable. For example, Willard (Willard, H.F., 1985, Am J Hum Genet, 37; Willard, H.F., 1991, Curr Opin Genet Dev. 1) found sequences of the same monomer in different higher order repeat units that showed a level of similarity approaching 99%. Further, there appear to be a significant number of off-target hits to other chromosomes. For example, bioinformatics research revealed that 14 oligonucleotide sequences derived from plasmid p17H8 (comprising the higher order repeat units in the centromere region of CHR17) had high homology to several other chromosomes (*e.g.,* chromosome 1, X, 11, 9, 20, 22, etc.). Although a number of sequences of each oligonucleotide had high homology (85-100%) to CHR17, there were also many off-target hits. For instance, a representative oligonucleotide (M2.1) had 21 on-target hits but also had 33

hits on chromosome 1; another oligonucleotide (M2.2) had 18 on-target hits but also had 14 hits on chromosome X (See FIG. 15). These results suggest that the centromere region of CHR17 may not contain sufficiently specific sequences for targeting. Indeed, examining the centromere region from a bioinformatics perspective indicates that designing probes uniquely specific to the centromere, which would be capable of providing selective signal without the use of blocking DNA, is not reasonable or expected to be possible.

[0013] Another reason that workers in the field expected it was not possible to make short, single-stranded CHR17 probes specific enough to CHR17 (*e.g.,* specific enough to eliminate the need for blocking DNA) is because of the lack of robustness of a single (or a few number of) single-stranded oligonucleotide probe(s). As discussed above, human CHR17-specific alpha satellite contains a higher order repeat unit that consists of 16 monomers, and each individual human being may carry different combinations of these monomers and their related variants (Waye JS and Willard HF, Molecular and Cellular Biology, Sept. 1986, p. 3156-3165). A single oligonucleotide probe, *e.g.* the 42mer described above, or even a few number of oligonucleotides covering a small number of monomers, may not be robust enough to detect CHR17 polymorphism in a human population (Waye JS, Willard HF., NAC 1986; 14(17); Willard, H.F. et al, 1987, Genomics, 1; Warburton, P.E. and Willard, H.F., 1995, J. Mol. Evol., 41). Indeed, a single CHR17-specific oligonucleotide probe (79mer) did not show equivalent (or better) sensitivity to the p17H8 plasmid derived probe. In particular, when the single 79mer CHR17 oligonucleotide was compared to the commercial probe (p17H8 probe), it was found that it passed (signal intensity ≥2, coverage ≥50%, and background<2) only 41.5% (113/272) at 1 µg/mL, 1hr compared to 61.1% (148/242) at 0.75 µg/mL, 6hr. Accordingly, the Chr17 Oligonucleotide (a single 79mer) failed to show equivalent sensitivity to the commercial probe design.

[0014] Another reason that workers in the field expected it was not possible to make short, single-stranded CHR17 probes specific enough for CHR17 was because the making of such oligonucleotide probes is very cumbersome and the manufacturability of such product is heretofore, not readily known. In particular, to span a 1 million bp genomic region with probes hybridizing to at least 60 kb of target, as many as 1200 unique 50-mer oligonucleotide probes may be needed. Manufacturing 1200 unique probes and combining them within a single reagent is difficult, expensive, and breaks new ground from a regulatory perspective.

SUMMARY

[0015] A set of 14 unique single-stranded probes that are highly specific for CHR17 and are highly robust enough to account for polymorphisms in a human population were created and synthesized. These single-stranded probes are fully compatible for use for the detection of HER2. In fact, these newly discovered oligonucleotide probes are so highly specific that the inventors were able to eliminate the use of blocking DNA in the assays disclosed herein. Furthermore, it was surprisingly discovered that these oligonucleotide probes have enhanced hybridization efficiency, which requires a significantly reduced hybridization time. These single-stranded oligonucleotide probes to CHR17 also enabled discrete enumerable rounded signals that are superior to those previously available. In particular, the detectable signals contrast to the nick-translation labeled double-stranded probes, which tend to generate signals with a wide range of sizes and shapes.

[0016] The single-stranded oligonucleotide probes to CHR17 of the present invention may be used in combination with one or more target probes directed to a target gene of interest. This allows for gene copy enumeration (e.g., determination of the ratio of a target gene to its corresponding chromosome), which may be important for tissue diagnostics. Alterations in DNA copy number are the hallmark of many types of cell proliferative disorders such as cancer. Indeed, some investigators have hypothesized that these are thought to drive some cancer pathogenesis processes. Representative alterations include large chromosomal gains and losses in addition to smaller scale amplifications and deletions. Considering that genomic instability may trigger the activation of oncogenes and/or the silencing of tumor suppressors, mapping regions of genomic aberrations is a useful tool to identify cancer-related genes. Such information - genomic aberrations - may provide useful information relative to diagnosis of cancer or as a prognostic aide. As mentioned above, HER2 is a gene found on CHR17; the present invention also features the use of single-stranded oligonucleotide probes to detect (and enumerate gene copy number) the HER2 gene on CHR17 in combination with CHR17 detection and enumeration using the aforementioned single-stranded oligonucleotide probes.

[0017] Accordingly, in an embodiment, a system for *in situ* detection of a control region of human chromosome 17 is provided, said system comprising a set of two or more single-stranded control probes specific for X distinct monomers of an alpha satellite control region of human chromosome 17, wherein X = 2-14, the control probes are each labeled with at least one first label, wherein each control probe comprises a sequence selected from the group consisting of SEQ ID NOs: 3-16; or a sequence selected from the group consisting of a truncated version of SEQ ID NOs: 3-16, the truncated version being at least 40 contiguous bp of said SEQ ID NOs:3-16; or a sequence selected from the group consisting of a sequence that has at least 70% sequence identity to one of SEQ ID NOs: 3-16, or complements thereof.

[0018] In another embodiment, a kit comprising a vessel containing the aforementioned system is provided.

[0019] In another embodiment, a slide is provided, the slide comprising a plurality of nuclei chromogenically stained for chromosome 17, wherein the slide is made using the aforementioned system.

[0020] In another embodiment, a method for *in situ* hybridization of a tissue sample is provided, the method comprising contacting the tissue sample with the aforementioned system.

[0021] In another embodiment, a method for dual bright-field *in situ* hybridization is provided, the method comprising contacting a tissue sample with a set of two or more single-stranded control probes specific for X distinct monomers of an alpha satellite control region of human chromosome 17, wherein X = 2-14, wherein each control probe is labeled with at least one first label and comprises a sequence selected from the group consisting of SEQ ID NOs: 3-16; or a sequence selected from the group consisting of a truncated version of SEQ ID NOs: 3-16, the truncated version being at least 40 contiguous bp of said SEQ ID NOs:3-16; or a sequence selected from the group consisting of a sequence that has at least 70% sequence identity to one of SEQ ID NOs: 3-16, or complements thereof; contacting the tissue sample with a single-stranded target probe

specific for a target region near or around the HER2 gene locus of human chromosome 17, the target probe being labeled with at least one second label; hybridizing the probes under conditions for a period of time less than about 3 hours; rinsing the sample to remove unbound probe; and staining the sample to detect hybridized probes.

[0022] In another embodiment, a method of scoring for a chromosome for HER2 gene copy number is provided, said method comprising obtaining a tissue sample having undergone *in situ* hybridization according to any of claims 13-16, wherein a control probe specific for human chromosome 17 and a target probe specific for HER2 are used; identifying an area of neoplastic nuclei with most copy numbers; and counting enumerable signals for HER2 signal in at least 20 nuclei; and calculating the ratio of HER2 signal to chromosome 17 signal (HER2/CHR17 ratio).

[0023] Additional features of the present disclosure will become apparent to those skilled in the art upon consideration of the following detailed description of illustrative embodiments exemplifying the best mode of carrying out the disclosure as presently perceived.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024]

FIG. 1(A-B) is a sequence (SEQ. ID. NO: 1) showing label locations and structural perspective of a disclosed probe showing an illustrative labeling approach.

FIG. 2(A-C) is a sequence (SEQ. ID. NO: 2) and structural perspectives of a disclosed probe.

FIG. 3(A-D) are graphs (A) and (B) and photomicrographs (C) and (D) which show HER2 signal intensity and coverage for probes as disclosed herein compared to a commercially available probe (labeled HER2PMA).

FIG. 4(A-B) are photomicrographs of stained breast tissue.

FIG. 5(A-B) are graphs showing HER2 staining for different hybridization conditions.

FIG. 6(A-D) are graphs and photomicrographs showing Chr17 signal intensity and background for particularly tested oligonucleotides.

FIG. 7(A-D) are graphs showing Chr17 signal intensity, staining coverage, background, and pass/fail for a single strand probe versus a double strand commercial probe product.

FIG. 8 is a photomicrograph of staining of a chromosomal metaphase spread showing specificity.

FIG. 9 is a series of graphs showing the effect of using 48%, 72%, and 100% of the 1196 HER2 oligonucleotide probes on intensity, coverage, and background.

FIG. 10 is a series of graphs showing no consistent linkage between longer hybridization times (*e.g.* 2 and 6hr) and improved staining intensity.

FIG. 11 (A-B) are photomicrographs of a breast tissue stained with a DISH assay.

FIG. 12(A-B) are photomicrographs of a lung tissue stained with a DISH assay.

FIG. 13(A-B) are photomicrographs of a gastric tissue stained with a DISH assay.

FIG. 14(A-D) are graphs showing (A) a weak signal for CHR17 using the 42-mer CHR17 oligonucleotide probe (B) that a 42-mer Chr17 oligonucleotide probe has weaker staining than the p17H8 probe at 33.2% formamide and increasing the concentration and hybridization time did not increase the signal with 33.2% formamide, (no 6 hr hybridization time point was performed as previous data suggested no difference between 1 to 6 hr hybridization time), (C) that 22.8% formamide gave a better signal for the 42-mer, but it was still weaker than PMA, and (D) that the stringency wash temperature for CHR17 oligonucleotide (42-mer) is not compatible with the HER2 oligonucleotide probes (68-72 °C).

FIG. 15 shows that 14 oligonucleotide sequences comprising the higher order repeat units in the centromere region of CHR17 had high homology to several other chromosomes (*e.g.,* chromosome 1, X, 11, 9, 20, 22, etc.). For example, Oligonucleotide M2.1 had 21 on-target hits but also had 33 hits on chromosome 1; Oligonucleotide M2.2 had 18 on-target hits but also had 14 hits on chromosome X.

FIG. 16(A-D) shows examples of concentric circles and simple closed curves used for evaluating enumerable signals. The schematic helps describe a generally round shape as described herein.

SEQUENCES

[0025] The nucleic acid sequences provided herein are shown using standard letter abbreviations for nucleotide bases. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. In the provided sequences:
SEQ ID NOs: 1-16 are examples of nucleic acid sequences of probes, e.g., probes with labels, to human chromosome 17.

DETAILED DESCRIPTION

I. Definitions

[0026] Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which a disclosed invention belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. "Comprising" means "including." Hence "comprising A or B" means "including A" or "including B" or "including A and B."

[0027] Suitable methods and materials for the practice and/or testing of embodiments of the disclosure are described below. Such methods and materials are illustrative only and are not intended to be limiting. Other methods and materials similar or equivalent to those described herein can be used. For example, conventional methods well known in the art to which the disclosure pertains are described in various general and more specific references, including, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Sambrook et al., Molecular Cloning: A Laboratory Manual, 3d ed., Cold Spring Harbor Press, 2001; Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates, 1992 (and Supplements to 2000); Ausubel et al., Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, 4th ed., Wiley & Sons, 1999; Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1990; and Harlow and Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1999.

[0028] Although methods and materials similar or equivalent to those described herein can be used to practice or test the disclosed technology, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

[0029] In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:
Conjugating, joining, bonding or linking: Covalently linking one molecule to another molecule to make a larger molecule. For example, making two polypeptides into one contiguous polypeptide molecule, or covalently attaching a mass tag, hapten, nucleic acid, or other molecule to a polypeptide, such as a scFv antibody.

[0030] **Contacting** refers to placement that allows association between two or more moieties, particularly direct physical association, for example both in solid form and/or in liquid form (for example, the placement of a biological sample, such as a biological sample affixed to a slide, in contact with a composition, such as a solution containing the probes disclosed herein).

[0031] **Detect:** To determine if an agent (such as a signal or particular antigen, protein or nucleic acid) is present or absent, for example, in a sample. In some examples, this can further include quantification, and/or localization, for example localization within a cell or particular cellular compartment. "Detecting" refers to any method of determining if something exists, or does not exist, such as determining if a target molecule is present in a biological sample. For example, "detecting" can include using a visual or a mechanical device to determine if a sample displays a specific characteristic. In certain examples, light microscopy and other microscopic means are used to detect a detectable label bound to or proximally to a target.

[0032] **Detectable label:** A molecule or material that can produce a detectable (such as visually, electronically or otherwise) signal that indicates the presence and/or concentration of a target, such as a target molecule, in a sample, such as a tissue sample. When conjugated to a molecule capable of binding directly or proximally to a target, the detectable label can be used to locate and/or quantify the target. Thereby, the presence and/or concentration of the target in a sample can be detected by detecting the signal produced by the detectable label. A detectable label can be detected directly or indirectly, and several different detectable labels conjugated to different

molecules can be used in combination to detect one or more targets. Multiple detectable labels that can be separately detected can be conjugated to different molecules that bind directly or proximally to different targets to provide a multiplexed assay that can provide detection of the multiple targets in a sample. Specific, non-limiting examples of labels include fluorescent and fluorogenic moieties, chromogenic moieties, haptens, affinity tags, and radioactive isotopes. The label can be directly detectable (e.g., optically detectable) or indirectly detectable (for example, via interaction with one or more additional molecules that are in turn detectable). Exemplary labels in the context of the probes disclosed herein are described below. Methods for labeling nucleic acids, and guidance in the choice of labels useful for various purposes, are discussed, e.g., in Sambrook and Russell, in Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press (2001) and Ausubel et al., in Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Intersciences (1987, and including updates).

[0033] Hapten: A molecule, typically a small molecule that can combine specifically with an antibody, but typically is substantially incapable of being immunogenic except in combination with a carrier molecule.

[0034] HER2: Also known as v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ErbB2), human epidermal growth factor receptor 2, Her2/neu, c-erb B2/neu, and neuroblastoma/glioblastoma derived oncogene homolog; GenBank Gene ID Accession No. 2064. A member of the epidermal growth factor receptor tyrosine kinase family. Her2 heterodimerizes with other ligand-bound EGF receptor family members, though it lacks a ligand binding domain and cannot bind ligands itself. Amplification and/or overexpression of Her2 occur in several types of cancer, including breast and ovarian cancer.

[0035] Her2 nucleic acid and protein sequences are publicly available. For example, the Her2 gene is located on chromosome 17q12 and its sequence is disclosed as GenBank Accession No. NC_000017.10(37844167-37884915). GenBank Accession Nos. NM_001005862, NM_004448, XM_005257139, and XM_005257140 disclose Her2 nucleic acid sequences, and GenBank Accession Nos.: NP_001005862, NP_004439, XP_005257196, and XP_005257197 disclose Her2 protein sequences, as provided by GenBank on October 4, 2013.

[0036] Hybridization: To form base pairs between complementary regions of two strands of DNA, RNA, or between DNA and RNA, thereby forming a duplex molecule. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (such as the Na+ concentration) of the hybridization buffer will determine the stringency of hybridization. The presence of a chemical which decreases hybridization (such as formamide) in the hybridization buffer will also determine the stringency (Sadhu et al., J. Biosci., 6:817-821, 1984). Calculations regarding hybridization conditions for attaining particular degrees of stringency are discussed in Sambrook et al., (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, NY (chapters 9 and 11). Hybridization conditions for ISH are also discussed in Landegent et al., Hum. Genet., 77:366-370, 1987; Lichter et al., Hum. Genet., 80:224-234, 1988; and Pinkel et al., Proc. Natl. Acad. Sci. USA, 85:9138-9142, 1988.

[0037] Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein, or cell) has been substantially separated or purified away from other biological components in a preparation, a cell of an organism, or the organism itself, in which the component occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins and cells. Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins. In some examples, the nucleic acid probes disclosed herein are isolated nucleic acid probes.

[0038] Linker: As used herein, a linker is a molecule or group of atoms positioned between two moieties. For example, a mass tag conjugate may include a linker between the mass tag and the specific binding moiety. Typically, linkers are bifunctional, *i.e.,* the linker includes a functional group at each end, wherein the functional groups are used to couple the linker to the two moieties. The two functional groups may be the same, *i.e.,* a homobifunctional linker, or different, *i.e.,* a heterobifunctional linker.

[0039] Multiplex, -ed, -ing: Embodiments of the present invention allow multiple targets in a sample to be detected substantially simultaneously, or sequentially, as desired, using plural different conjugates. Multiplexing can include identifying and/or quantifying nucleic acids generally, DNA, RNA, peptides, proteins, both individually and in any and all combinations. Multiplexing also can include detecting two or more of a gene, a messenger and a protein in a cell in its anatomic context.

[0040] Probe: A nucleic acid molecule that is capable of hybridizing with a target nucleic acid molecule (*e.g.,* genomic target nucleic acid molecule) and, when hybridized to the target, is capable of being detected either directly or indirectly. Thus probes permit the detection, and in some examples quantification, of a target nucleic acid molecule. In particular examples, a probe includes at least two segments complementary to uniquely specific nucleic acid sequences of a target nucleic acid molecule and are thus capable of specifically hybridizing to at least a portion of the target nucleic acid molecule. Generally, once at least one segment or portion of a segment has (and remains) hybridized to the target nucleic acid molecule other portions of the probe may (but need not) be physically constrained from hybridizing to those other portions' cognate binding sites in the target (*e.g.,* such other portions are too far distant from their cognate binding sites); however, other nucleic acid molecules present in the probe can bind to one another, thus amplifying signal from the probe. A probe can be referred to as a "labeled nucleic acid probe," indicating that the probe is coupled directly or indirectly to a detectable moiety or "label," which renders the probe detectable.

[0041] Sample: A specimen containing DNA (for example, genomic DNA), RNA (including mRNA), protein, or combinations thereof, obtained from a subject. Examples include, but are not limited to, chromosomal preparations, peripheral blood, urine, saliva, tissue biopsy, fine needle aspirate, surgical specimen, bone marrow, amniocentesis samples, and autopsy material. In one example, a sample includes genomic DNA. In some examples, the sample is a cytogenetic preparation, for example which can be placed on microscope slides. In particular examples, samples are used directly, or can be manipulated prior to use, for example, by fixing (*e.g.,* using formalin).

[0042] **Sequence identity:** The identity (or similarity) between two or more nucleic acid sequences is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are.

[0043] Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math., 2:482, 1981; Needleman & Wunsch, J. Mol. Biol., 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. USA, 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS 5:151-3, 1989; Corpet et al., Nuc. Acids Res., 16:10881-90, 1988; Huang et al. Computer Appls. in the Biosciences, 8:155-65, 1992; and Pearson et al., Meth. Mol. Bio., 24:307-31, 1994. Altschul et al., J. Mol. Biol., 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

[0044] The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403-10, 1990) is available from several sources, including the National Center for Biotechnology and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

[0045] BLASTN may be used to compare nucleic acid sequences, while BLASTP may be used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

[0046] The BLAST-like alignment tool (BLAT) may also be used to compare nucleic acid sequences (Kent, Genome Res. 12:656-664, 2002). BLAT is available from several sources, including Kent Informatics (Santa Cruz, CA) and on the Internet (genome.ucsc.edu).

[0047] Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1554 nucleotides is 75.0 percent identical to the test sequence (1166÷1554*100=75.0). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 20-nucleotide region that aligns with 15 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is, 15÷20*100=75).

[0048] Subject: Any multi-cellular vertebrate organism, such as human or non-human mammals (e.g., veterinary subjects).

[0049] Target nucleic acid sequence or molecule: A defined region or particular portion of a nucleic acid molecule, for example a portion of a genome (such as a gene or a region of mammalian genomic DNA containing a gene of interest). In an example where the target nucleic acid sequence is a target genomic sequence, such a target can be defined by its position on a chromosome (e.g., in a normal cell), for example, according to cytogenetic nomenclature by reference to a particular location on a chromosome; by reference to its location on a genetic map; by reference to a hypothetical or assembled contig; by its specific sequence or function; by its gene or protein name; or by any other means that uniquely identifies it from among other genetic sequences of a genome. In some examples, the target nucleic acid sequence is mammalian genomic sequence (for example human genomic sequence).

[0050] In some examples, alterations of a target nucleic acid sequence (e.g., genomic nucleic acid sequence) are "associated with" a disease or condition. In some examples, detection of the target nucleic acid sequence can be used to infer the status of a sample with respect to the disease or condition. For example, the target nucleic acid sequence can exist in two (or more) distinguishable forms, such that a first form correlates with absence of a disease or condition and a second (or different) form correlates with the presence of the disease or condition. The two different forms can be qualitatively distinguishable, such as by polynucleotide polymorphisms, and/or the two different forms can be quantitatively distinguishable, such as by the number of copies of the target nucleic acid sequence that are present in a cell.

[0051] Uniquely specific sequence: A nucleic acid sequence (for example, a sequence of at least of at least 20 bp (such as at least 20 bp, 30 bp, 40 bp, 50 bp, 60 bp, 70 bp, 80 bp, 90 bp, 100 bp, or more) that is present only one time in a haploid genome of an organism. In a particular example, a uniquely specific nucleic acid sequence is a nucleic acid sequence from a target nucleic acid that has 100% sequence identity with the target nucleic acid and has no significant identity to any other nucleic acid sequences present in the specific haploid genome that includes the target nucleic acid.

[0052] Vector: Any nucleic acid that acts as a carrier for other ("foreign") nucleic acid sequences that are not native to the vector. When introduced into an appropriate host cell a vector may replicate itself (and, thereby, the foreign nucleic acid sequence) or express at least a portion of the foreign nucleic acid sequence. In one context, a vector is a linear or circular nucleic acid into which a nucleic acid sequence of interest is introduced (for example, cloned) for the purpose of replication (*e.g.,* production) and/or manipulation using standard recombinant nucleic acid techniques (*e.g.,* restriction digestion). A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements known in the art. Common vectors include, for example, plasmids, cosmids, phage, phagemids, artificial chromosomes (*e.g.,* BAC, PAC, HAC, YAC), and hybrids that incorporate features of more than one of these types of vectors. Typically, a vector includes one or more unique restriction sites (and in some cases a multi-cloning site) to facilitate insertion of a target nucleic acid sequence.

II. Systems for in situ Hybridization for Chromosome Enumeration

[0053] The present disclosure describes an automated bright-field dual ISH assay for the simultaneous detection of a gene target (*e.g.* HER2) and a centromere target (*e.g.* CHR17) using single-strand oligonucleotide probes. One aspect of this assay is the discovery of particular probes that enable compatibility between the centromere probe and the gene probe. In particular, a pool of single-strand oligonucleotide probes for the centromere targets was discovered that are highly compatible with a pool of single-strand oligonucleotide probes for the gene target. The centromere oligonucleotide sequences are selected to avoid the need for using human blocking DNA. The probes, as used in a dual *in situ* hybridization (DISH) assay achieved comparable staining performance to commercial dual-strand probe products; however, the single-strand probes hybridize in 1 hour while the dual-strand probes required longer (*e.g.* 6 hours). The two probe types were highly concordant on the diagnosis of gene status, but the single-strand probe achieved a lower assay failure rate. When tested on specimens with unknown pre-analytical conditions and tissue quality, the single-strand probe proved to be more robust than dual-strand probe products even using the highly disparate hybridization times (*e.g.* 1 hour versus 6 hours).

[0054] Gene copy number assessment is a major ISH application in both cytogenetics and anatomical pathology laboratories. For example, determination of HER2 gene status requires the use of chromosome 17 centromere (CEN 17) enumeration, so the HER2/CEN 17 ratio can be calculated. In order to take advantages of the single-strand oligonucleotide probe approach for this application, however, several technical hurdles had to be overcome. First, CHR17 oligonucleotide probe needs to accommodate the assay conditions for HER2 oligonucleotide probe; Second, CHR17 oligonucleotide probe needs to be robust enough for adequate sensitivity; Third, CHR17 oligonucleotide probe needs to be specific enough to CHR17 centromere and therefore there is no need for the suppressive hybridization reagents such as human placenta or Cot1 DNA.

[0055] Currently, all commercially available HER2 ISH assays use labeled segments of double strand DNA obtained from bacterial artificial chromosome (BAC) as the original source (*See* HER2 FISH PHARMDX Kit Interpretation guide - breast cancer, PATHVYSION HER2 DNA Probe Kit, and Interpretation Guide Ventana INFORM HER2 Dual ISH DNA Probe Cocktail Assay). BACs are either directly labeled with fluorophore molecules as probes (HER2 FISH PHARMDX Kit, Dako and PATHVYSION HER2 DNA Probe Kit, Abbott Molecular, Inc.), or as template to generate more specific sequences by physical subtraction or avoidance of repetitive sequences (SPOT-LIGHT HER2 CISH Kit, Life Technologies, Inc. and INFORM HER2 Dual ISH assay, Ventana Medical Systems, Inc.). It is well known that these double strand probes require prolonged hybridization time (*i.e.* from 6hrs to 18hrs) to ensure sufficient hybridization to the targets. The extended time reflects low hybridization efficiency. Importantly, it has a negative impact on patients who must wait for their diagnosis for because of the extended turnaround times associated with tissue-based ISH assays. The criteria in TABLE 1 are typically used to evaluate whether a particular DISH assay is acceptable or not acceptable.

TABLE 1. Analytical slide scoring criteria.

	Acceptable (A)	Not Acceptable (N)
Signal Intensity	**3,** Signals are bright and easily identified in > 80% of cells within the target region.	**1,** Specific signals are visible but too weak to reliably identify in ≥ 50% of the targeted region.
	2, Specific signals are sufficiently intense to reliably identify in > 50% of cells within the targeted region.	**0.5,** Signals are visible but absent or too weak to reliably identify in 80% of cells.
		0, Signals are not visible.
Background	**1,** Background signals (either punctate signals or diffuse, hazy staining) are present but are sufficiently weak in intensity within the nuclei to permit reliable identification of specific signals in > 50% of cells within the target region.	**3,** Background signals (punctate signals, diffuse staining, haze) cover 75-100% of cells within the target region and are sufficiently intense to obscure specific signals.
	0, Background staining is not observed in > 80% of cells within the target region.	**2,** Background signals (punctate signals, diffuse staining, haze) cover 50-75% of cells within the target region and are sufficiently intense to obscure specific signals

[0056] There have been several approaches to enhance the hybridization efficiency, so as to decrease the turnaround times for these assays. One approach for accelerating hybridization reaction rates was to change the composition of the hybridization buffer. Currently, formamide is routinely used to lower the melting point and annealing temperature of nucleic acid strands. The benefit of lowering the temperature is to better preserve the tissue morphology (*See* McConaughy BL, et al., Biochemistry 8: 3289-3295 (1969) and Blake RD, Delcourt SG, Nucleic Acids Res 24: 2095-2103 (1996)). However, a long hybridization is required to obtain sufficient signal intensity as formamide reduces hybridization rate. Recently, Matthiesen SH et al., PLoS One. 2012; 7(7) reported ethylene carbonate (EC) as the substitute for formamide in hybridization buffers with the effect of reducing FISH hybridization time to one hour. It is understood that this technology underlies the new commercial product HER2 IQFISH PHARMDX (Dako).

[0057] Another approach has been to switch from double strand to single strand probes. Single strand probes are understood to have higher sensitivity than that of double strand probes, presumably because a proportion of the denatured double-strand probe renatures to form probe homoduplexes, thus preventing their hybridization to genomic targets in the test samples (See Taneja K and Singer RH, ANALYTICAL BIOCHEMISTRY 166.389-398 (1987), Lewis ME, et al., Peptides. 6 Suppl 2:75-87 (1985) and Strachan T, Read AP. Human Molecular Genetics. 2nd edition. New York: Wiley-Liss (1999)).

[0058] In Kourilsky P, et al., Biochimie. 56 (9): 1215-21 (1974), it was found that the percentage of single strand nucleotides (available as probe) is inversely proportional to the amount of competitive strand nucleotide in the solution at the pre-hybridization step. A mathematical model developed in

this study revealed that homologous competition is a powerful competitor of DNA-target hybridization. Several laboratories have reported that single-strand probes provide higher sensitivity on hybridization than double-stranded probes (See An SF, et al., Mol Cell Probes. Jun;6(3):193-200 (1992), Hannon K, et al,. Anal Biochem. Aug 1;212(2):421-7 (1993), and Cox KH, et al., Dev Biol. Feb;101(2):485-502 (1984)). In particular, An *et al.*'s work demonstrated digoxigenin (DIG) labeled single-strand probes were at least two-fold more sensitive than double-strand DIG PCR-labeled probes of the same size, and 10-fold more sensitive than nick translated double-strand probes of the same size in dot-blot hybridization. In ISH application, single stranded probes were more sensitive, *i.e.* detecting approximately two- to four-fold the number of infected cells than double-strand probes of the same size. Furthermore, it gave much less background staining than double-stranded probe of the same size in ISH. Single-strand probes did not need purification before use in ISH; in contrast, the double strand PCR probes needed purification; otherwise there was a large amount of nonspecific background staining. Further, it was demonstrated by Hannon *et al.* that the DIG-labeled single strand DNA probe was approximately 27% more intense (by an image analysis program) than that obtained using DIG-double strand probe. Cox KH et al., Dev Biol. Feb;101 (2):485-502 (1984) found eightfold more of the single strand probe hybridized to target sequence at apparent saturation, while the observed hybridization reaction with double strand probes terminated at a level far below saturation of available target sites. This implied that most of the double stand probe was removed from the ISH reaction relatively early. Consistent to the above findings, we discovered single strand HER2/CHR17 probes with 1 hour hybridization achieved comparable staining performance to that of dual strand probe with 6 hour hybridization. Surprisingly, the single strand probe with 1 hour hybridization also demonstrated superior robustness on a cohort of difficult tissues (TMA). Our data is aligned with previous observations that single strand probes tend to have higher hybridization efficiency than that of double strand probes.

[0059] While not being limited to a particular theory, we perceive another advantage of single strand probes being that that they more easily penetrate the tissue. Double strand probes are usually labeled by incorporating labeled dNTPs in an enzymatic DNA synthesis reaction. The labeled probes are sized to smaller fragments by DNase treatment or mechanical sonication. The optimal length of the labeled ISH probes is typically understood to be between 100 and 400 nucleotides according to Cox, et al., Dev Biol. Feb;101(2):485-502 (1984) and Haase *et al.* (*See* Haase, A. et al., in Methods in Virology (Maramorosch, K., and Koprowski, Eds.), Vol. 7, pp. 189-226, Academic Press, San Diego, CA (1984)). However, the "random" nature of the size-down process for the labeled probes is understood to render the majority of the probes within the correct size, but produce a wide population of sizes. Single strand probes generated by oligonucleotide synthesis have well-defined short lengths which facilitate the ability of the probe to penetrate tissue better than larger double strand probes, especially on difficult tissue specimens (e.g. over-fixed). It was discovered that the single strand probes described herein exhibit superior staining on a cohort of difficult tissues (TMA), which may be partially explained by better tissue penetration of short and uniform probes.

[0060] Furthermore, from the perspective of manufacturing and quality control, a single strand probe having an exact structure are more reproducibly manufactured using oligonucleotide synthesis compared to the approaches based on PCR, nick translation, or other random synthetic approaches.

[0061] Oligonucleotide probes ideally hybridize maximally with the target and minimally with non-targets (See Li X, et al., Nucleic Acids Res., Oct 24; 33(19): 6114-23 (2005)). While these references applicable to solution or array based hybridization may be relevant to consider, the hybridization kinetics to genomic targets on formalin fixed paraffin embedded (FFPE) tissues is highly unpredictable in comparison. This unpredictability is understood to be imparted by the highly complex and variable nature of human tissues, especially in comparison to either a solution or an array. In microarray application, a 50-mer probe showing 75% identity to non-targets or with 15-, 20-, or 35-base stretches showed cross-reactivity in Kane MD, et al., Nucleic Acids Res. Nov 15;28(22):4552-7 (2000). A 60-mer probe with 80% identity to non-targets showed cross-reactivity to non-target in Hughes TR, et al., Nat Biotechnol. Apr;19(4]:342-7 (2001). Similar results were shown with a 70-mer by Wang X, Seed B., Bioinformatics. May 1;19(7):796-802 (2003).

[0062] Li X, et al., Nucleic Acids Res., Oct 24; 33(19): 6114-23 (2005) appears to have proposed an optimal choice for designing 50-mer oligonucleotides: identity of <87%, continuous stretch of < 17 bases, and free energy of >29 kcal/mol. Both 50-mer and 70-mer probes were observed to have minimal cross-hybridization to sequences having less than 85% identity to the respective targets, whereas the signal intensity increased substantially for probes that had more than 90% identity to the respective targets (*See* He Z, et al., Appl Environ Microbiol. Jul;71 (7):3753-60 (2005)). He Z *et al.* suggested that a gene-specific probe should have an identity of <85% to non-targets under the conditions examined.

[0063] While synthetic oligonucleotide probes have been widely used for messenger RNA ISH, it has not been used on genomic targets until recently (*See* Bergstrom Lucas A, Ruvolo M, Kulkarni V, Chen S, Mullinax B, Venneri J, Barboza J, Happe S, Fulmer-Smentek S, Srinivasan M. Designing Custom Oligonucleotide FISH Probes for the Detection of Chromosomal Rearrangements in FFPE Tissues. American Society of Human Genetics 2013 Meeting*)*. Bergstrom *et al.* reported SUREFISH probes with fluorescence labels that were understood to include thousands of unique oligonucleotides. The oligonucleotide sequences were tiled across the targeted chromosomal region of translocation breakpoints for the detection of chromosomal rearrangements. A short hybridization time (75min) was reported for these probes.

[0064] The most common target of chromosome 17 ISH is the centromeric regions. The centromeric regions of all human chromosomes are characterized by distinct subsets of a diverse tandemly repeated DNA family, alpha satellite. The fundamental unit of alpha satellite is the diverged 171-bp monomer, by which higher-order chromosome-specific repeat units are organized. The human chromosome 17-specific alpha satellite contains approximately 1,000 polymorphic higher-order repeat units that range from 11 to 16 monomers. The predominant form of chromosome 17 alpha satellites is a ~ 2,700 base pair repeat unit that consists of 16 monomers, which is present in 500 to 1,000 copies per chromosome 17. Since alpha satellite DNA clusters most often contain monomer variants that differ from the consensus sequence by up to 40% (Rosandić M, Paar V, Gluncić M, Basar I, Pavin N, Croat Med J. 2003 Aug; 44(4):386-406), blocking DNA is usually included with the probes to suppress sequences contained within the target loci that are common to other chromosomes. One aspect of the present disclosure is the discovery of single strand oligonucleotides from the 2,700 base pair repeat unit with comparable melting temperature (Tm) range to that of a 80-mer single strand gene probe. In particular, it was

discovered that 14 particular single strand oligonucleotides specific to the chromosome 17 centromere could robustly enable a gene/centromere DISH assay with the 80-mer gene probes. The sequences of the 14 oligonucleotides are from 10 of the 16 monomers; therefore they increase the probability of recognizing haplotype-specific sequence variation in the population.

[0065] While the examples herein describe particularly a single strand oligonucleotide-based CHR17 (or HER2/CHR17 dual) ISH assay, it is understood that those of ordinary skill in the art could apply the discoveries disclosed herein to any gene/centromere combination of interest.

[0066] Difficulties frequently encountered in both IHC and ISH testing results from the manner in which the tissues are typically preserved. The mainstay of the diagnostic pathology laboratory has been for many decades the formalin-fixed, paraffin-embedded block of tissue, sectioned and mounted upon glass slides. Fixation in such a preservative causes cross-linking of macromolecules, both amino acids and nucleic acids. These cross-linked components must be removed to allow access of the probe to the target nucleic acid and to allow the antibody to recognize the corresponding antigen. "Unmasking" the antigen and/or nucleic acid is typically accomplished manually with multiple pretreatment, proteolytic digestion, and wash steps. Prior to staining, complete removal of the paraffin is also required so that it does not interfere with antibody or probe binding. Deparaffinization may be achieved by the use of multiple (*e.g.,* two or three) successive clearing reagents that are paraffin solvents (*e.g.,* xylene, xylene substitutes, or toluene).

[0067] In an example, preparing includes the step of cell conditioning. Cell conditioning is discussed in greater detail in U.S. Patent 6,855,552, Towne, et al. "Automated immunohistochemical and *in situ* hybridization assay formulations". In illustrative cell conditioning steps, a cell conditioning reagent is applied and the sample is contacted at the appropriate temperature for an appropriate duration of time so that the antigens and/or nucleic acid targets are sufficiently expressed for detection. One aspect of the present disclosure is that the automated instrument can automatically adjust the cell conditioning duration and/or temperature in response to the user inputs. Cell conditioning may further include applying a protease reagent. Illustratively, a protease treatment may involve the step of contacting a protease solution to a biological sample. The protease treatment, as with cell conditioning, is intended to increase the expression of target antigens and/or nucleic acids.

[0068] Exemplary cell conditioning reagents include, for nucleic acid targets (ISH), a solution including ethylenediaminetetraacetic acid (EDTA) may be used. The contacting may be done at a temperature of about 95° C for between about 2 and about 90 minutes. For protein targets (IHC), a cell conditioning solution may be a boric acid buffer. The contacting may be may be done at a temperature of about 100° C for between about 2 and about 90 minutes. A partial list of possible reagents appears in Analytical Morphology, Gu, ed., Eaton Publishing Co. (1997) at pp. 1-40. Sodium dodecyl sulfate (SDS) and/or ethylene glycol may be included in the conditioning solution. Furthermore, metal ions or other materials may be added to these reagents to increase effectiveness of the cell conditioning. Exemplary cell conditioning solutions are available from Ventana Medical Systems, Inc., Tucson, AZ (Cell Conditioning 1 (CC1) catalog #: 950-124; Cell Conditioning 2 (CC2) catalog #: 950-123; SSC (10X) catalog #: 950-110; ULTRA Cell Conditioning (ULTRA CC1) catalog #: 950-224; ULTRA Cell Conditioning (ULTRA CC2) catalog #: 950-223,

Protease 1 catalog #: 760-2018; Protease 2 catalog #: 760-2019; Protease 3 catalog #: 760-2020). Applying the immunohistochemical binding reagent or the *in situ* hybridization binding reagent may occur subsequent to applying the cell conditioning reagent and prior to applying the chromogenic reagent.

[0069] The method may include applying a rinsing reagent. Between various steps described herein and as part of the system described herein, rinse steps may be added to remove unreacted residual reagents from the prior step. Rinse steps may further include incubations, which include maintaining a rinsing reagent on the sample for a pre-determined time at a pre-determined temperature with or without mixing. The conditions appropriate for the rinsing steps may be distinct between the various steps. Exemplary rinsing reagents are available from Ventana Medical Systems, Inc., Tucson, AZ (Reaction Buffer (10x) catalog #: 950-300; Special Stains Wash (10x) catalog #: 860-015).

[0070] Exemplary automated systems available through Ventana Medical Systems, Inc., Tucson, AZ include SYMPHONY® Staining System, catalog #: 900-SYM3, VENTANA® BenchMark Automated Slide Preparation Systems, catalog #s: N750-BMKXT-FS, N750-BMKU-FS, VENTANA, and VENTANA® BenchMark Special Stains automated slide stainer. These systems employ a microprocessor controlled system including a revolving carousel supporting radially positioned slides. A stepper motor rotates the carousel placing each slide under one of a series of reagent dispensers positioned above the slides. Bar codes on the slides and reagent dispensers permits the computer controlled positioning of the dispensers and slides so that different reagent treatments can be performed for each of the various tissue samples by appropriate programming of the computer.

A. CHROMOSOME 77

[0071] As previously discussed, the most common target for a control region of chromosome 17 (CHR17) ISH is the centromeric region. The centromeric regions of all human chromosomes are characterized by distinct subsets of a diverse tandemly repeated DNA family, alpha satellite. Since alpha satellite DNA clusters most often contain monomer variants that differ from the consensus sequence by up to 40%, blocking DNA is usually included with the probes to suppress sequences contained within the target loci that are common to other chromosomes.

[0072] We designed single-stranded probes directed to the control region (centromeric region) of chromosome 17 that achieved acceptable signal intensity levels and background levels within 1 hour of hybridization and without the use of blocking DNA (*See* TABLE 3 of Example 1). For example, the probes are configured to achieve a staining intensity of greater than or equal to 2 and staining coverage of greater than or equal to 50% of nuclei. We also designed single-stranded probes directed to a target region near and within the HER2 gene locus that also achieved acceptable signal intensity levels and background levels within 1 hour of hybridization and without the use of blocking DNA.

[0073] From the perspective of manufacturing and quality control, a single-stranded probe having an exact structure are more reproducibly manufactured using oligonucleotide synthesis compared

to the approaches based on PCR, nick translation, or other random synthetic approaches. From the perspective of cost analysis, the probes that do not require blocking DNA provide for a less expensive assay.

[0074] The present disclosure describes systems for ISH featuring a control probe specific to a control region of a chromosome, e.g., a centromere target of a chromosome. The chromosome detected may be chromosome 17, or any other appropriate chromosome. The control probe is configured to achieve a staining intensity of greater than or equal to 2 and staining coverage of greater than or equal to 50% of the number of nuclei within 3 hours when applied to a control sample *(e.g.,* as described above, TABLE 1). In some examples, a staining coverage of ≥55% of the number of nuclei within 3 hours is achieved, e.g., ≥60% of the number of nuclei, ≥65% of the number of nuclei, ≥70% of the number of nuclei, ≥75% of the number of nuclei, ≥80% of the number of nuclei, ≥85% of the number of nuclei, ≥90% of the number of nuclei.

[0075] In some embodiments, the systems for ISH also feature a target probe specific for a target region (*e.g.,* for detecting a target gene) on the corresponding chromosome.

[0076] The control probe may comprise a first plurality (*e.g.,* a plurality of a single probe, a plurality of different probes such as a set or pool of probes) of single-stranded oligonucleotide probes. One or more of the plurality of probes may comprise a sequence selected from the group consisting of SEQ ID NOs: 3-16 (See TABLE 3 below). In some examples, one or more of the first plurality of probes comprise a truncated version (*e.g.,* at least 30 contiguous bp, at least 35 contiguous bp, at least 40 contiguous bp, at least 45 contiguous bp, at least 50 contiguous bp, at least 55 contiguous bp, at least 60 contiguous bp, at least 65 contiguous bp, at least 70 contiguous bp, at least 75 contiguous bp, etc.) of one of the sequences in TABLE 3 (SEQ ID NOs: 3-16). In some examples, one or more of the first plurality of probes comprises a sequence that has at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, or at least 95% sequence identity to one of the sequences in TABLE 3 (SEQ ID NOs: 3-16). The first plurality of single-stranded oligonucleotide probes is configured to hybridize uniquely and specifically to a portion of the control region of human chromosome 17 so that other chromosomes or portions thereof are not evidently labeled.

[0077] As used herein, reference to use of SEQ ID NOs: 3-16 may also include the use of complementary sequences of SEQ ID NOs: 3-16.

[0078] In some examples, the probes target between 2 and 16 distinct portions within the control region. In some examples, the probes target between 4 and 16 distinct portions within the control region. In some examples, the probes target between 6 and 16 distinct portions within the control region. In some examples, the probes target between 8 and 16 distinct portions within the control region. In some examples, the probes target between 10 and 16 distinct portions within the control region. In some examples, the probes target between 12 and 16 distinct portions within the control region. In some examples, the probes target between 14 and 16 distinct portions within the control region. In some examples, the probes target between 2 and 12 distinct portions within the control region. In some examples, the probes target between 4 and 12 distinct portions within the control region. In some examples, the probes target between 6 and 12 distinct portions within the control region. In some examples, the probes target between 8 and 12 distinct portions within the control

region. In some examples, the probes target between 10 and 12 distinct portions within the control region.

[0079] Without wishing to limit the present invention to any theory or mechanism, it is believed that the probes may be able to identify at least 60% of chromosome 17 polymorphisms, at least 70% of chromosome 17 polymorphisms, at least 80% of chromosome 17 polymorphisms, at least 90% of chromosome 17 polymorphisms, at least 95% of chromosome 17 polymorphisms, at least 99% of chromosome 17 polymorphisms, etc. It is not clear how many monomers would need to be probed to be sufficient for identifying at least 60% of chromosome 17 polymorphisms, at least 70% of chromosome 17 polymorphisms, at least 80% of chromosome 17 polymorphisms, at least 90% of chromosome 17 polymorphisms, at least 95% of chromosome 17 polymorphisms, at least 99% of chromosome 17 polymorphisms, etc.

[0080] The first plurality of single-stranded oligonucleotide probes may be constructed in a variety of lengths. For example, the probes may each comprise between 40 to 100 nucleotides. In some examples, the probes each comprise between 50 to 100 nucleotides. In some examples, the probes each comprise between 60 to 110 nucleotides. In some examples, the probes each comprise between 40 to 120 nucleotides. In some examples, the probes each comprise at least 40 nucleotides. In some examples, the probes each comprise at least 50 nucleotides. In some examples, the probes each comprise at least 60 nucleotides. In some examples, the probes each comprise at least 70 nucleotides.

[0081] The present invention also features slides with a plurality of nuclei chromogenically stained for chromosome 17. The slide may be contacted with one or more of the above systems *(e.g.,* probes). The slide may feature enumerable signals indicative of the number of chromosome 17 centromere regions present in a cell, *e.g.,* cells should exhibit two copies of the CHR17 centromere normally.

[0082] In some examples, more than 50% of the nuclei have enumerable signals for the chromosome. An enumerable signal may be a generally round shape. The round shape can be defined as shown in FIG. 16, wherein a round shape is a simple closed curve that fits within a first region, the first region lies on and outside an inner circle and on and inside a concentric outer circle, the inner circle has an inner radius (R_{in}) and the outer circle has a outer radius (R_{out}), wherein the simple close curve has a radius R_{simple}, wherein $R_{in} \leq R_{simple} \leq R_{out}$, and wherein, R_{in} is $\geq$ 50% of R_{out}. A simple closed curve is a curve that does not cross itself and ends at the same point where it begins.

[0083] In some examples, the inner radius is no less than 40% of the outer radius. In some examples, the inner radius is no less than 50% of the outer radius. In some examples, the inner radius is no less than 55% of the outer radius. In some examples, the inner radius is no less than 60% of the outer radius. In some examples, the inner radius is no less than 65% of the outer radius. In some examples, the inner radius is no less than 70% of the outer radius. In some examples, the inner radius is no less than 75% of the outer radius. In some examples, the inner radius is no less than 80% of the outer radius. In some examples, the inner radius is no less than 85% of the outer radius. In some examples, the inner radius is no less than 90% of the outer radius.

[0084] In some examples, more than 60% of the nuclei have enumerable signals for the chromosome. In some examples, more than 70% of the nuclei have enumerable signals for the chromosome. In some examples, more than 80% of the nuclei have enumerable signals for the chromosome. In some examples, more than 90% of the nuclei have enumerable signals for the chromosome. The nuclei may not be enumerable if the tissue sectioning process has destroyed that portion of the cell, if that portion of the cell is divided between two slides, or if that portion of the cell is wholly within a separate slide. The nuclei may also be enumerable if the tissue condition prevents probe penetration to the specific binding site (*i.e.* the cell is not sufficiently accessible to the probe) or if the target region of DNA is substantially degraded.

[0085] In some examples, the sum of the surface area covered by staining signal is calculated and assigned a 100% value, and at least 50% of the sum of the surface area is derived from discrete round signals (or round shapes).

[0086] A round shape can be defined as shown in FIG. 16, wherein a round shape is a simple closed curve that fits within a first region, the first region lies on and outside an inner circle and on and inside a concentric outer circle, the inner circle has an inner radius (R_{in}) and the outer circle has a outer radius (T_{out}), wherein the simple close curve has a radius R_{simple}, wherein $R_{in} \leq R_{simple} \leq R_{out}$, and wherein, R_{in} is $\geq$ 50% of R_{out}.

[0087] In some examples, the inner radius is no less than 50% of the outer radius. In some examples, more than 60% of said sum of the surface area is derived from discrete round signals. In some examples, more than 70% of said sum of the surface area is derived from discrete round signals. In some examples, the inner radius is no less than 60% of the outer radius. In some examples, the inner radius is no less than 75% of the outer radius. In some examples, the inner radius is no less than 90% of the outer radius.

[0088] In some examples, the outer radius is between about 0.25 to 0.675 µm. In some examples, the outer radius is between about 0.2 to 0.75 µm. In some examples, the outer radius is between about 0.15 to 1 µm. In some examples, the average outer radius of the enumerable signals is between about 0.2 to 0.75 µm. In some examples, the average outer radius of the enumerable signals has a standard deviation of less than 0.5 µm. In some examples, the average outer radius of the enumerable signals has a standard deviation of less than 0.25 µm.

[0089] In some examples, the enumerable round signals are mono-sized. As used herein, a population of "mono-sized" round signals have the R_{simple} being within 15% plus or minus of each other. In some examples, the population of "mono-sized" round signals have the R_{simple} being within 10% plus or minus of each other. In some examples, the population of "mono-sized" round signals have the R_{simple} being within 5% plus or minus of each other.

B. TARGET GENE (HER2)

[0090] In some embodiments, the systems for ISH also feature a target probe specific for a target

region (*e.g.,* for detecting a target gene, for gene copy enumeration) on the corresponding chromosome.

[0091] The target region may comprise the HER2 gene locus (or nearby nucleotides). Disclosed herein are probes directed to the human HER2 gene (See Terms for GenBank accession numbers). As described below in detail in EXAMPLE 1, the HER2 target probe is specific to a region between nucleotides 35,027,979 and 35,355,516 of human chromosome 17.

[0092] In some examples, the target probe comprises a second plurality (*e.g.,* a plurality of a single probe, a plurality of different probes such as a set or pool of probes) of single-stranded oligonucleotide probes. The second plurality of single-stranded oligonucleotide probes is configured to hybridize uniquely and specifically to a portion of the target region of the corresponding chromosome so that other genes or chromosomes or portions thereof are not evidently labeled.

[0093] The present disclosure also features means of making the control region of chromosome 17 visible. In some examples, the means of making the control region of chromosome 17 visible comprises the step of contacting the probes with a detection reagent specific to the probes. Detection reagents are well known in the art. For example, the detection reagent may comprise an antibody or other probe, which binds to the control probe. The detection reagent may comprise a molecule (*e.g.,* enzyme, substrate, tag) that makes the first label of the probe visible. The detection reagent may comprise a plurality of reagents effective for making the probe visible (*e.g.,* more than one antibody, enzyme, substrate, chromogen, etc.). In some embodiments, the detection reagent emits a color. Additional detection reagents (labels, tags, enzymes, substrates, chromogens, antibodies, etc.) are further disclosed herein. The present disclosure also features means of visualizing the control region of chromosome 17, wherein the probe (*e.g.,* first label) is made visible by a detection reagent and the visibility of the first label is indicative of the control region of chromosome 17. Means for visualizing labeled probes are well known to one of ordinary skill in the art. For example, the means for visualizing the control region of chromosome 17 comprises may be a microscope (*e.g.,* bright field microscope, fluorescence microscope, inverted microscope). In some examples, the means for visualizing the control region of chromosome 17 comprises a luminometer. In some examples, the means for visualizing the control region of chromosome 17 comprises a radiometric detection machine (*e.g.,* gamma counter, etc.). In some examples, the means for visualizing the control region of chromosome 17 comprises a spectrometer. In some examples, the means for visualizing the control region of chromosome 17 comprises a real-time PCR machine. In some examples, the means for visualizing the control region of chromosome 17 comprises a scintillation and/or luminescence counter. In some examples, the means for visualizing the control region of chromosome 17 comprises a colorimeter. Other means for visualizing the control region of chromosome 17 are known in the art.

C. KITS

[0094] Also disclosed are kits including one or more of the oligonucleotide probes (for example, one or more of SEQ ID NOs: 3-16). For example, kits can include at least one probe (such as at

least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more probes) or at least one probe set (such as at least 1, 2, 3, 4, or 5 probe sets) as described herein. In one example, the kit comprises probes such as at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or all of SEQ ID NOs: 3-16 (or sequences at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% identical to SEQ ID NOs: 3-16; or truncated versions of SEQ ID NOs: 3-16). In other examples, the probes (or the probe set) are in a single container.

[0095] The kits may also comprise one or more reagents for detecting the probe (for example, by *in situ* hybridization), or for producing a detectably labeled probe. For example, a kit can include at least one of the disclosed nucleic acid probes or probe sets, along with one or more buffers, labeled dNTPs, a labeling enzyme (such as a polymerase), primers, nuclease free water, and instructions for producing a labeled probe. In another example, the kit includes one or more of the disclosed nucleic acid probes (unlabeled or labeled) along with buffers and other reagents for performing *in situ* hybridization. For example, if one or more unlabeled probes are included in the kit, labeling reagents can also be included, along with specific detection agents (for example, fluorescent, chromogenic, luminescent and/or radiometric) and other reagents for performing an *in situ* hybridization assay, such as paraffin pretreatment buffer, protease(s) and protease buffer, prehybridization buffer, hybridization buffer, wash buffer, counterstain(s), mounting medium, or combinations thereof. In some examples, such kit components are present in separate containers. The kit can optionally further include control slides (such as positive or negative controls) for assessing hybridization and signal of the probe(s).

[0096] In certain examples, the kits include avidin, antibodies, and/or receptors (or other anti-ligands). Optionally, one or more of the detection agents (including a primary detection agent, and optionally, secondary, tertiary or additional detection reagents) are labeled, for example, with a hapten or fluorophore (such as a fluorescent dye or quantum dot). In some instances, the detection reagents are labeled with different detectable moieties (for example, different fluorescent dyes, spectrally distinguishable quantum dots, different haptens, etc.). For example, a kit can include two or more nucleic acid probes or probe sets that correspond to and are capable of hybridizing to different target nucleic acids (for example, any of the target nucleic acids disclosed herein). The first probe or probe set can be labeled with a first detectable label (e.g., hapten, fluorophore, etc.), the second probe or probe set can be labeled with a second detectable label, and any additional probes or probe sets (*e.g.,* third, fourth, fifth, etc.) can be labeled with additional detectable labels. The first, second, and any subsequent probes or probe sets can be labeled with different detectable labels, although other detection schemes are possible. If the probe(s) are labeled with indirectly detectable labels, such as haptens, the kits can include detection agents (such as labeled avidin, antibodies or other specific binding agents) for some or all of the probes. In one example, the kit includes probes and detection reagents suitable for multiplex ISH.

[0097] In one example, the kit also includes an antibody conjugate, such as an antibody conjugated to a label (*e.g.,* an enzyme, fluorophore, or fluorescent nanoparticle). In some examples, the antibody is conjugated to the label through a linker, such as PEG, 6X-His, streptavidin, or GST.

D. DETECTABLE LABLES AND METHODS OF LABELING

[0098] The probes disclosed herein may comprise one or more labels (*e.g.,* at least 1 at least 2, at least 3, at least 4, at least 5, at least 6, etc.), for example to permit detection of the probe/nucleic acid sequence (or region) of interest. In various applications, such as *in situ* hybridization procedures, a nucleic acid probe includes a label (*e.g.,* a detectable label). A "detectable label" is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or quantity (for example, gene copy number) of a target nucleic acid (to which the labeled uniquely specific nucleic acid molecule is bound or hybridized) in a sample. The disclosure is not limited to the use of particular labels, although examples are provided.

[0099] A label associated with one or more nucleic acid molecules (such as the disclosed probes) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

[0100] Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies. Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Patent No. 5,866,366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2'disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl]maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); 2',7' -difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho-cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and

succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives.

[0101] Other suitable fluorophores include thiol-reactive europium chelates, which emit at approximately 617 nm (Heyduk and Heyduk, Analyt. Biochem., 248:216-27, 1997; J. Biol. Chem., 274:3315-22, 1999), as well as GFP, Lissamine™, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Patent No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Carlsbad, CA) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Patent Nos. 5,696,157, 6,130,101 and 6, 716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Patent Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Patent No. 5,132,432) and Marina Blue (U.S. Patent No. 5,830,912). In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, *e.g.,* a quantum dot. Additional labels include, for example, radioisotopes (such as 3H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd3+, and liposomes.

[0102] Detectable labels that can be used with nucleic acid molecules (such as the disclosed probes) also include enzymes, for example horseradish peroxidase (HRP), alkaline phosphatase (AP), acid phosphatase, glucose oxidase, β-galactosidase, β-glucuronidase, or β-lactamase. Where the detectable label includes an enzyme, a chromogen, fluorogenic compound, or luminogenic compound can be used in combination with the enzyme to generate a detectable signal (numerous of such compounds are commercially available, for example, from Life Technologies). Particular examples of chromogenic compounds include diaminobenzidine (DAB), 4-nitrophenylphosphate (pNPP), fast red, fast blue, bromochloroindolyl phosphate (BCIP), nitro blue tetrazolium (NBT), BCIP/NBT, AP Orange, AP blue, tetramethylbenzidine (TMB), 2,2'-azino-di-[3-ethylbenzothiazoline sulphonate] (ABTS), o-dianisidine, 4-chloronaphthol (4-CN), nitrophenyl-β-D-galactopyranoside (ONPG), o-phenylenediamine (OPD), 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal), methylumbelliferyl-β-D-galactopyranoside (MU-Gal), p-nitrophenyl-α-D-galactopyranoside (PNP), 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 3-amino-9-ethyl carbazol (AEC), fuchsin, iodonitrotetrazolium (INT), tetrazolium blue, and tetrazolium violet.

[0103] Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver *in situ* hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redox-active agent reduces the metal ion, causing it to form a detectable precipitate (*See,* for example, U.S. Patent No. 7,632,652,). Metallographic

detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate (*See,* for example, U.S. Patent No. 6,670,113).

[0104] In non-limiting examples, the disclosed nucleic acid probes are labeled with dNTPs covalently attached to hapten molecules (such as a nitro-aromatic compound (*e.g.,* 2,4-dinitrophenyl (DNP)), biotin, fluorescein, digoxigenin (DIG), etc.). Additional haptens suitable for labeling the disclosed probes include nitropyrazole, 3-hydroxyquinoxaline, thiazolesulfonamide, nitrocinnamic acid, rotenone, 7-(diethylamino)coumarin-3-carboxylic acid, benzodiazepine, or benzofuran haptens (*See, e.g.,* International Pat. Publ. No. WO 2012/003476). Methods for conjugating haptens and other labels to dNTPs *(e.g.,* to facilitate incorporation into labeled probes) are well known in the art. For examples of procedures, *see, e.g.,* U.S. Patent Nos. 5,258,507, 4,772,691, 5,328,824, and 4,711,955. Indeed, numerous labeled dNTPs are available commercially, for example from Life Technologies (Carlsbad, CA). A label can be directly or indirectly attached to a dNTP at any location on the dNTP, such as a phosphate (*e.g.,* α, β or γ phosphate) or a sugar.

[0105] Detection of labeled nucleic acid molecules can be accomplished by contacting the hapten-labeled nucleic acid molecules bound to the genomic target nucleic acid with a primary anti-hapten antibody. In one example, the primary anti-hapten antibody (such as a mouse anti-hapten antibody) is directly labeled with an enzyme. In another example, a secondary anti-species antibody (such as a goat anti-mouse IgG antibody) conjugated to an enzyme is used for signal amplification. In chromogenic *in situ* hybridization CISH a chromogenic substrate is added, for SISH, silver ions and other reagents as outlined in the referenced patents/applications are added.

[0106] In some examples, a probe is labeled by incorporating one or more labeled dNTPs using an enzymatic (polymerization) reaction. For example, the disclosed nucleic acid probes (for example, incorporated into a plasmid vector) can be labeled by nick translation (using, for example, biotin, DNP, digoxigenin, etc.) or by random primer extension with terminal transferase (e.g., 3' end tailing). In some examples, the nucleic probe is labeled by a modified nick translation reaction where the ratio of DNA polymerase I to deoxyribonuclease I (DNase I) is modified to produce greater than 100% of the starting material. In particular examples, the nick translation reaction includes DNA polymerase I to DNase I at a ratio of at least about 800:1, such as at least 2000:1, at least 4000:1, at least 8000:1, at least 10,000:1, at least 12,000:1, at least 16,000:1, such as about 800:1 to 24,000:1 and the reaction is carried out overnight (for example, for about 16-22 hours) at a substantially isothermal temperature, for example, at about 16°C to 25°C (such as room temperature). If the probe is included in a probe set (for example, multiple plasmids, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, or more plasmids), the plasmids may be mixed in an equal molar ratio prior to performing the labeling reaction (such as nick translation or modified nick translation).

[0107] In other examples, chemical labeling procedures can also be employed. Numerous reagents (including hapten, fluorophore, and other labeled nucleotides) and other kits are commercially available for enzymatic labeling of nucleic acids, including the disclosed nucleic acid probes. As will be apparent to those of skill in the art, any of the labels and detection procedures disclosed above are applicable in the context of labeling a probe, *e.g.,* for use in *in situ* hybridization reactions. For example, the Amersham MULTIPRIME® DNA labeling system, various

specific reagents and kits available from Molecular Probes/Life Technologies, or any other similar reagents or kits can be used to label the nucleic acids disclosed herein. In particular examples, the disclosed probes can be directly or indirectly labeled with a hapten, a ligand, a fluorescent moiety (*e.g.,* a fluorophore or a semiconductor nanocrystal), a chromogenic moiety, or a radioisotope. For example, for indirect labeling, the label can be attached to nucleic acid molecules via a linker (*e.g.,* PEG or biotin). Additional methods that can be used to label probe nucleic acid molecules are provided in U.S. Pat. No. 7,541,455.

E METHODS FOR in situ HYBRIDIZATION FOR CHROMOSOME ENUMERATION

[0108] The present invention also features *in situ* hybridization (ISH) assays, *e.g.,* bright-field ISH assays, for detection of a gene target and a chromosome (*e.g.,* centromere target of a chromosome) using single-strand oligonucleotide probes. For example, a method comprises contacting a tissue sample with a control probe specific to a control region of a chromosome (*e.g.,* chromosome 17), wherein the control probe is a single-stranded oligonucleotide probe labeled with at least one first label. The control probe may be configured to achieve a staining intensity of ≥2 and staining coverage of ≥50% of nuclei within 3 hours when applied to a control sample. The method further comprises hybridizing the control probe to the control region under conditions for a period of time less than about 3 hours (*e.g.*, ≤ about 2.5 hours, ≤ about 2 hours, ≤ about 1.5 hour, or ≤ about 1 hour), rinsing the sample to remove unbound probe, and detecting the presence of the hybridized probe.

[0109] In some embodiments, the method further comprises contacting the tissue sample with a target probe specific to a target region (*e.g.,* HER2) of the chromosome, wherein the target probe is a single-stranded oligonucleotide probe labeled with at least one second label.

[0110] In some embodiments, the method further comprises applying chromogenic detection reagents that recognize the first label and amplifying the signal associated with said first label. The method may feature the use of one or more probes (*e.g.,* SEQ ID NOs: 3-16) or systems as described herein.

[0111] Genome-specific blocking DNA (such as human DNA, for example, total human placental DNA or Cot-1™ DNA) is usually included in a hybridization solution (such as for *in situ* hybridization) to suppress probe hybridization to repetitive DNA sequences or to counteract probe hybridization to highly homologous (frequently identical) off target sequences when a probe complementary to a human genomic target nucleic acid is utilized. In hybridization with standard probes, in the absence of genome-specific blocking DNA, an unacceptably high level of background staining (for example, non-specific binding, such as hybridization to non-target nucleic acid sequence) is usually present, even when a "repeat-free" probe is used. The disclosed nucleic acid probes exhibit reduced background staining, even in the absence of blocking DNA. In particular examples, the hybridization solution including the disclosed probes does not include genome-specific blocking DNA (for example, total human placental DNA or Cot-1™ DNA, if the probe is complementary to a human genomic target nucleic acid). This advantage is derived from the uniquely specific nature of the target sequences included in the nucleic acid probe; each labeled probe sequence binds only to the cognate uniquely specific genomic sequence. This

results in dramatic increases in signal to noise ratios for ISH techniques.

[0112] As such, some methods herein may be free from the use of blocking DNA. However, in some examples, blocking DNA may be used. In some examples, an amount of blocking DNA is used but the amount of blocking DNA is sufficient to block out no more than a specified percent of the non-specific binding, *e.g.,* no more than 50%, 40%, 30%, 20%, or 10%.

[0113] In order to determine an amount of blocking DNA that is sufficient to block out no more than a specified percent (*e.g.,* 50%) of the non-specific binding, the following tests may be conducted. Set up an *in situ* hybridization assay, contact a tissue sample with a double strand control probe specific to a control region of a chromosome (in combination with zero to a serially, gradually increasing amount of blocking DNA); hybridize the double strand control probe to the control region; rinse the sample to remove unbound double strand probe; and detect the presence of the hybridized probe. Then observe the amount of background that is blocked by the serially increasing blocking DNA in each assay. The amount of blocking DNA that achieves a specified percent of the blocking of the background corresponds to the amount of blocking DNA that is sufficient to block out no more than a specified percent (*e.g.,* 50%) of the non-specific binding. For example, the amount of blocking DNA that achieves blocking out 50% of percent of the background corresponds to the amount of blocking DNA that is sufficient to block out no more than 50% of the non-specific binding.

[0114] In some examples, said amount of blocking DNA is between about 1 pg/ml to 1 mg/ml. In some examples, said amount of blocking DNA is between about 1 pg/ml to 0.5 mg/ml. In some examples, said amount of blocking DNA is between about 1 pg/ml to 0.25 mg/ml. In some examples, said amount of blocking DNA is between about 1 pg/ml to 1 µg/ml.

[0115] Methods for obtaining two bright-field chromogenic *in situ* hybridization signals per cell may comprise contacting a tissue sample containing a plurality of cells with a control probe specific to a control region of a single chromosome, the probe selected so as to not evidently bind non-specifically in the absence of blocking DNA; hybridizing the control probe to the control region of said chromosome; rinsing the sample to remove unbound probe; and detecting the presence of the hybridized probe via a chromogenic reagent so as to generate two bright-field chromogenic *in situ* hybridization signals per cell. In order to determine that the selected probe does not evidently bind non-specifically in the absence of blocking DNA, a comparative assay (Assay 2) may be conducted along side with the aforementioned assay (Assay 1), wherein the same selected probe is employed in both Assay 1 and Assay 2. Assay 1 is free of the blocking DNA and Assay 2 employs a blocking DNA. Then the respective data of the two assays are compared. The selected probe does not evidently bind non-specifically in the absence of blocking DNA when the data of the two respective assays are the same or substantially the same.

[0116] In some examples the hybridization solution may contain carrier DNA from a different organism (for example, salmon sperm DNA or herring sperm DNA, if the genomic target nucleic acid is a human genomic target nucleic acid) to reduce non-specific binding of the probe to non-DNA materials (for example to reaction vessels or slides) with high net positive charge which can non-specifically bind to the negatively charged probe DNA.

[0117] Methods of the present disclosure may comprise detecting signals wherein more than 50% of the nuclei of the tissue sample have enumerable signals for said chromosome, wherein an enumerable signal is a generally round shape *(e.g.,* as described above). In some examples, background signals are not observed in > 70% of cells of the tissue sample. In some examples, background signals are not observed in > 80% of cells of the tissue sample. In some examples, background signals are not observed in > 90% of cells of the tissue sample. In some examples, background signals are present but are sufficiently weak in intensity so as to permit identification of enumerable signals in > 50% of the nuclei.

[0118] In some examples, more than 60% of the nuclei have enumerable chromosome signals. In some examples, more than 70% of the nuclei have enumerable chromosome signals. In some examples, the inner radius is no less than 60% of the outer radius. In some examples, the inner radius is no less than 75% of the outer radius. In some examples, the inner radius is no less than 90% of the outer radius.

[0119] *In situ* hybridization (ISH) involves contacting a sample containing a target nucleic acid *(e.g.,* a genomic target nucleic acid) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid (for example, one or more of the probes disclosed herein). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The chromosome sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the target is performed using standard techniques.

[0120] For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat anti-avidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (*e.g.,* in alkaline phosphatase (AP) buffer). The enzyme reaction can be performed in, for example, AP buffer containing NBT/BCIP and stopped by incubation in 2 X SSC. For a general description of *in situ* hybridization procedures, *see, e.g.,* U.S. Patent No. 4,888,278.

[0121] Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Patent Nos. 5,447,841; 5,472,842; and 5,427,932; CISH is described in U.S. Patent No. 6,942,970, and additional detection methods are provided in U.S. Patent No. 6,280,929,.

[0122] Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above, probes labeled with fluorophores (including fluorescent dyes and quantum dots)

can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a non-fluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, courmarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (*e.g.,* the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (*e.g.,* quantum dot) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (*e.g.,* secondary or specific antibodies), which can in turn be labeled with a fluorophore. Optionally, the detectable label is attached directly to the antibody, receptor (or other specific binding agent).

[0123] Alternatively, the detectable label is attached to the binding agent via a linker, such as a hydrazide thiol linker, a polyethylene glycol linker, or any other flexible attachment moiety with comparable reactivities. For example, a specific binding agent, such as an antibody, a receptor (or other anti-ligand), avidin, or the like can be covalently modified with a fluorophore (or other label) via a heterobifunctional polyalkyleneglycol linker such as a heterobifunctional polyethyleneglycol (PEG) linker. A heterobifunctional linker combines two different reactive groups selected, *e.g.,* from a carbonyl-reactive group, an amine-reactive group, a thiol-reactive group and a photo-reactive group, the first of which attaches to the label and the second of which attaches to the specific binding agent.

[0124] In other examples, the probe, or specific binding agent (such as an antibody, *e.g.,* a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (*e.g.,* as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (*e.g.,* binding reagents) and chemistries (*e.g.,* linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/0117153.

[0125] In further examples, a signal amplification method is utilized, for example, to increase sensitivity of the probe. For example, tyramide signal amplification may be utilized (*See* U.S. Pat. No. 5,196,306). In one variation of this method a biotinylated nucleic acid probe detects the presence of a target by binding thereto. Next a streptavidin-peroxidase conjugate is added. The streptavidin binds to the biotin. A substrate of biotinylated tyramide (tyramine is 4-(2-aminoethyl)phenol) is used, which presumably becomes a free radical when interacting with the peroxidase enzyme. The phenolic radical then reacts quickly with the surrounding material, thus depositing or fixing biotin in the vicinity. This process is repeated by providing more substrate (biotinylated tyramide) and building up more localized biotin. Finally, the "amplified" biotin deposit is detected with streptavidin attached to a fluorescent molecule. Alternatively, the amplified biotin deposit can be detected with avidin-peroxidase complex, that is then fed 3,3'-diaminobenzidine to produce a brown color. It has been found that tyramide attached to fluorescent molecules also serve as substrates for the enzyme, thus simplifying the procedure by eliminating steps. Yet

another amplification approach is described in U.S. Patent Publ. No. 2013/0260379.

[0126] In other examples, the signal amplification method utilizes branched DNA (bDNA) signal amplification. In some examples, target-specific oligonucleotides (label extenders and capture extenders) are hybridized with high stringency to the target nucleic acid. Capture extenders are designed to hybridize to the target and to capture probes, which are attached to a microwell plate. Label extenders are designed to hybridize to contiguous regions on the target and to provide sequences for hybridization of a preamplifier oligonucleotide. Signal amplification then begins with preamplifier probes hybridizing to label extenders. The preamplifier forms a stable hybrid only if it hybridizes to two adjacent label extenders. Other regions on the preamplifier are designed to hybridize to multiple bDNA amplifier molecules that create a branched structure. Finally, alkaline phosphatase (AP)-labeled oligonucleotides, which are complementary to bDNA amplifier sequences, bind to the bDNA molecule by hybridization. The bDNA signal is the chemiluminescent product of the AP reaction (*See, e.g.,* Tsongalis, Microbiol. Inf. Dis., 126:448-453, 2006; U.S. Pat. No. 7,033,758).

[0127] In further examples, the signal amplification method utilizes polymerized antibodies. In some examples, the labeled probe is detected by using a primary antibody to the label (such as an anti-DIG or anti-DNP antibody). The primary antibody is detected by a polymerized secondary antibody (such as a polymerized HRP-conjugated secondary antibody or an AP-conjugated secondary antibody). The enzymatic reaction of AP or HRP leads to the formation of strong signals that can be visualized.

[0128] It will be appreciated by those of skill in the art that by appropriately selecting labeled probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acids (*e.g.*, genomic target nucleic acids) in a single assay (*e.g.*, on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target nucleic acid can be labeled with a first hapten, such as biotin, while a second probe that corresponds to a second target nucleic acid can be labeled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific binding agent (in this case avidin labeled with a first fluorophore, for example, a first spectrally distinct quantum dot, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labeled with a second fluorophore (for example, a second spectrally distinct quantum dot, *e.g.,* that emits at 705 nm)). Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

[0129] Additional details regarding certain detection methods, e.g., as utilized in CISH and SISH procedures, can be found in Bourne, The Handbook of Immunoperoxidase Staining Methods, published by Dako Corporation, Santa Barbara, CA.

[0130] Difficulties frequently encountered in ISH testing may result from the manner in which the tissues are typically preserved. The mainstay of the diagnostic pathology laboratory has been for many decades the formalin-fixed, paraffin-embedded block of tissue, sectioned and mounted upon

glass slides. Fixation in such a preservative causes cross-linking of macromolecules, both amino acids and nucleic acids. These cross-linked components must be removed to allow access of the probe to the target nucleic acid and to allow the antibody to recognize the corresponding antigen. "Unmasking" the antigen and/or nucleic acid is typically accomplished manually with multiple pretreatment, proteolytic digestion, and wash steps. Prior to or staining, complete removal of the paraffin is also required so that it does not interfere with antibody or probe binding. Deparaffinization may be achieved by the use of multiple (*e.g.*, two or three) successive clearing reagents that are paraffin solvents (*e.g.*, xylene, xylene substitutes, or toluene).

[0131] Preparing the sample may include the step of cell conditioning. Cell conditioning is discussed in greater detail in U.S. Patent 6,855,552, Towne, et al. "Automated immunohistochemical and *in situ* hybridization assay formulations". In illustrative cell conditioning steps, a cell conditioning reagent is applied and the sample is contacted at the appropriate temperature for an appropriate duration of time so that the antigens and/or nucleic acid targets are sufficiently expressed for detection. One aspect of the present disclosure is that the automated instrument can automatically adjust the cell conditioning duration and/or temperature in response to the user inputs. Cell conditioning may further include applying a protease reagent. Illustratively, a protease treatment may involve the step of contacting a protease solution to a biological sample. The protease treatment, as with cell conditioning, is intended to increase the expression of target antigens and/or nucleic acids.

[0132] Cell conditioning reagents such as ethylenediaminetetraacetic acid (EDTA) for nucleic acid targets (ISH) may be used. The contacting may be done at a temperature of about 95° C for between about 2 and about 90 minutes. A partial list of possible reagents appears in Analytical Morphology, Gu, ed., Eaton Publishing Co. (1997) at pp. 1-40. Sodium dodecyl sulfate (SDS) and/or ethylene glycol may be included in the conditioning solution. Furthermore, metal ions or other materials may be added to these reagents to increase effectiveness of the cell conditioning. Exemplary cell conditioning solutions are available from Ventana Medical Systems, Inc., Tucson, AZ (Cell Conditioning 1 (CC1) catalog #: 950-124; Cell Conditioning 2 (CC2) catalog #: 950-123; SSC (10X) catalog #: 950-110; ULTRA Cell Conditioning (ULTRA CC1) catalog #: 950-224; ULTRA Cell Conditioning (ULTRA CC2) catalog #: 950-223, Protease 1 catalog #: 760-2018; Protease 2 catalog #: 760-2019; Protease 3 catalog #: 760-2020). In some examples, applying the *in situ* hybridization binding reagent occurs subsequent to applying the cell conditioning reagent and prior to applying the chromogenic reagent.

[0133] In illustrative examples, the method includes applying a rinsing reagent. Between various steps described herein and as part of the system described herein, rinse steps may be added to remove unreacted residual reagents from the prior step. Rinse steps may further include incubations, which include maintaining a rinsing reagent on the sample for a pre-determined time at a pre-determined temperature with or without mixing. The conditions appropriate for the rinsing steps may be distinct between the various steps. Exemplary rinsing reagents are available from Ventana Medical Systems, Inc., Tucson, AZ (Reaction Buffer (10x) catalog #: 950-300; Special Stains Wash (10x) catalog #: 860-015).

[0134] Exemplary automated systems available through Ventana Medical Systems, Inc., Tucson, AZ include SYMPHONY® Staining System, catalog #: 900-SYM3, VENTANA® BenchMark

Automated Slide Preparation Systems, catalog #s: N750-BMKXT-FS, N750-BMKU-FS, VENTANA, and VENTANA® BenchMark Special Stains automated slide stainer. These systems employ a microprocessor controlled system including a revolving carousel supporting radially positioned slides. A stepper motor rotates the carousel placing each slide under one of a series of reagent dispensers positioned above the slides. Bar codes on the slides and reagent dispensers permits the computer controlled positioning of the dispensers and slides so that different reagent treatments can be performed for each of the various tissue samples by appropriate programming of the computer.

[0135] While the present invention describes a single-stranded oligonucleotide-based HER2/CHR17 dual ISH assay, it is understood that those of ordinary skill in the art could apply the discoveries disclosed herein to other gene/centromere combination of interest.

[0136] In some embodiments, the disclosed systems (e.g., probes) can be used in methods of determining the copy number of a target nucleic acid (such as HER2) in a biological sample (such as a tissue sample). Methods of determining the copy number of a gene or chromosomal region are well known to those of skill in the art. In some examples, the methods include *in situ* hybridization (such as fluorescent, chromogenic, or silver *in situ* hybridization), comparative genomic hybridization, or polymerase chain reaction (such as real-time quantitative PCR). In some examples, methods of determining gene copy number include counting the number of ISH signals (such as fluorescent, colored, or silver spots) for the target nucleic acid in one or more individual cells. The methods may also include counting the number of ISH signals (such as fluorescent, colored, or silver spots) for a reference (such as a chromosome-specific probe) in the cells. In particular examples, the number of copies of the gene (or chromosome) may be estimated by the person (or computer, in the case of an automated method) scoring the slide. In some examples, an increased copy number relative to a control (such as an increase of about 1.5-fold, 2-fold, 3-fold, 5-fold, 10-fold, 20-fold, or more relative to a control sample or reference value) indicates an increase in the target nucleic acid copy number.

[0137] In some examples, the method includes counting the number of copies per cell or nucleus of a reference, such as a chromosomal locus known not to be abnormal, for example a centromere. In some examples, the reference is on the same chromosome as the gene of interest. Exemplary reference chromosomes that can be used for particular human genes of interest are provided in TABLE 2. In particular examples, the reference locus is detected by using a centromere-specific probe. Such probes are known in the art and are commercially available, for example, Vysis CEP probes (Abbott Molecular, Des Plaines, IL) and SPOTLIGHT centromeric probes (Invitrogen, Carlsbad, CA). In some examples, a ratio of target nucleic acid copy number to reference copy number greater than about two (such as greater than about 2, 3, 4, 5, 10, 20, or more) indicates an increase in the target nucleic acid copy number.

TABLE 2. Exemplary reference chromosomes for particular target nucleic acids

Target Nucleic Acid	Reference Chromosome
PTEN	10
HER2	17
PIK3CA	3
TOP2A	17

Target Nucleic Acid	Reference Chromosome
MET	7
MDM2	12

F. METHODS OF SCORING

[0138] The present invention also features methods of scoring gene copy number of a target region and optionally comparing it to the copy number of a control region. For additional methods of scoring, which may be used with the methods described herein, reference is made to U.S. Publ. Appl. No. 2012/0141472 for disclosure related to scoring ISH.

[0139] In some examples, an increased gene copy number includes the gene copy number per nucleus (such as average gene copy number per nucleus) in the sample of greater than about two copies of the gene per nucleus (such as greater than 2, 3, 4, 5, 10, or 20 copies). In other examples, an increased gene copy number includes a ratio of gene copy number to its corresponding chromosome copy number (such as an average gene:chromosome ratio) in the sample of greater than about 2 (such as a ratio of greater than 2, 3, 4, 5, 10, or 20). In further examples, an increased gene copy number includes an increase in gene copy number relative to a control (such as an increase of about 1.5-fold, about 2-fold, about 3-fold, about 5-fold, about 10-fold, about 20-fold, or more). Therefore, in some examples, the method includes comparing the gene copy number in the sample from the subject to the gene copy number in a control or a reference value or range of values expected for the gene copy number in an appropriate normal tissue.

[0140] Also disclosed herein is a method of scoring (for example, enumerating) copy number of a gene in a sample from a subject, wherein the sample is stained by ISH (such as FISH, SISH, CISH, or a combination of two or more thereof) for the gene of interest and wherein individual copies of the gene are distinguishable in cells in the sample. In particular examples, the sample is a biological sample from a subject, such as a tumor sample (for example, a tumor biopsy). Methods of determining gene copy number by ISH are well known in the art.

[0141] In some examples, the method includes identifying individual cells in a sample with the highest number of signals per nucleus for the gene (such as the strongest signal in the sample), counting the number of signals for the gene in the identified cells, and determining an average number of signals per cell, thereby scoring the gene copy number in the sample. In additional examples, the method further includes counting the number of signals for a reference (such as a chromosomal locus known not to be abnormal, for example, centromeric DNA) and determining an average ratio of the number of signals for the gene to the number of signals for the reference per cell.

[0142] The scoring method may include identifying individual cells in the sample (such as a tissue section or tumor core) having the highest number of signals (such as the highest number of spots per cell or the brightest intensity of staining) for the gene of interest in the cells in the sample.

Thus, the disclosed method may not determine gene copy number in a random sampling of cells in the sample. Rather, the method may include specifically counting gene copy number in those cells that have the highest gene copy number in the sample. In some examples, identifying the individual cells having the highest number of signals for the gene includes examining a sample stained by ISH for the gene under low power microscopy (such as about 20> < magnification). Cells With the strongest signal (for example, highest amplification signal under higher power) are identified for counting by eye or by an automated imaging system. In some examples, such as when the sample is a tissue section, the sample is examined (for example, visually scanned) to identify a region that has a concentration of tumor cells that has amplification of the gene. Gene copy number in the cells with highest amplification in the selected region is then counted. In other examples, such as when the sample is a tumor core (such as a tumor microarray), most of the sample is visible in the field of view under low power magnification and the individual cells (such as tumor cells) with the strongest signal (for example, highest amplification signal under high power) are separately identified for counting. In particular examples, the cells chosen for counting the gene copy number may be non-consecutive cells, such as cells that are not adjacent to or in contact with one another. In other examples, at least some of the cells chosen for counting the gene copy number may be consecutive cells, such as cells that are adjacent to or in contact with one another.

[0143] The disclosed methods may include counting the number of ISH signals (such as fluorescent, colored, or silver spots) for the gene in the identified cells. The methods may also include counting the number of ISH signals (such as fluorescent, colored or silver spots) for a reference (such as a chromosome-specific probe) in the identified cells. In some examples, the number of spots per cells is distinguishable in the identified cells and the number of spots are counted (or enumerated) and recorded. In other examples, one or more of the identified cells may include a cluster, which is the presence of multiple overlapping signals in a nucleus that cannot be counted (or enumerated). In particular examples, the number of copies of the gene (or chromosome) may be estimated by the person (or computer, in the case of an automated method) scoring the slide. For example, one of skill in the art of pathology may estimate that a cluster contains a particular number of copies of a gene (such as 10, 20, or more copies) based on experience in enumerating gene copy number in a sample. In other examples, the presence of a cluster may be noted as a cluster, without estimating the number of copies present in the cluster.

[0144] The number of cells identified for counting is a sufficient number of cells that provides for detecting a change (such as an increase or decrease) in gene copy number. In some examples, the number of cells identified for counting is at least about 20, for example, at least 25, 30, 40, 50, 75, 100, 200, 500, 1000 cells, or more. In a particular example, about 50 cells are counted. In other examples, every cell in the sample or every cell in a microscope field of vision, or in a number of microscope fields (such as at least 2 microscope fields, at least 3, at least 4, at least 5, at least 6 microscope fields, and the like) which contains 3 or more copies of the gene of interest (such as 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more) is counted.

[0145] Methods may feature obtaining a sample having undergone ISH according to methods disclosed herein. An area of neoplastic nuclei with the most copy numbers is identified and the enumerable signals for the chromosome/target are counted in 50-100 neoplastic nuclei and either 50 adjacent mesenchymal nuclei or 50 adjacent normal epithelial nuclei.

[0146] Scoring criteria may be as follows: no staining or < 1 dot/ 10 cells is scored as 0; 1-3 dots/cell is scored as 1; 4-9 dots/cell, none or very few dot clusters is scored as 2; 10-15 dots/cell and < 10% dots are in clusters is scored as 3; and > 15 dots/cell and >10% dots are in clusters is scored as 4.

[0147] In some examples, the average number of target signals (e.g., HER2) per nuclei is calculated. In some examples, the average number of chromosome (e.g., CHR17) copies per nuclei is calculated. In some examples, the target signal to chromosome signal ratio is calculated.

[0148] The disclosure is further illustrated by the following non-limiting Examples.

EXAMPLES

Example 7

A. Specimens

[0149] Breast tissue samples were utilized for developing and optimizing the single strand oligonucleotide HER2 and/or CHR17 single and dual ISH assays. Samples were obtained from a tissue specimen archive maintained at Ventana Medical Systems, Inc. (Tucson, AZ). These samples were redundant clinical specimens that had been de-identified and unlinked from patient information and therefore patient informed consent was not required (6). Paraffin sections (4 µm) containing tissue cores of formalin-fixed, paraffin-embedded breast tissue were placed onto SUPERFROST Plus glass slides.

B. Probes

[0150] INFORM HER2 DUAL ISH DNA Probe reagent includes a probe dispenser that contains 12µg/ml of dinitrophenyl (DNP)-labeled HER2 probe cocktailed with a digoxigenin-labeled (DIG) Chr17 probe with 4mg/ml human blocking DNA in a formamide-based buffer.

[0151] The single strand oligonucleotide HER2 probe (HER2 oligonucleotide probe) is a dinitrophenyl (DNP)-labeled, repeat-free genomic probe specifically targeting the HER2 gene region. Similar to INFORM HER2 DUAL ISH DNA Probe, the HER2 oligonucleotide probe spans > 327,000 nucleotides (nt) (35,027,979 - 35,355,516) of genomic DNA from human Chromosome 17, encompassing the HER2 target region (UCSC Genome Browser on Human May 2004 (NCBI35/hg17) Assembly). The HER2 oligonucleotide sequences were designed from the sequences in INFORM HER2 DUAL ISH DNA Probe. Each of the HER2 oligonucleotides was designed with 80-mer length; hence stringency level for non-target binding was raised higher according to the aforementioned oligonucleotide probe design criteria. Specificity of the HER2

oligonucleotide probe was experimentally validated on metaphase spreads under the examined ISH assay conditions.

[0152] Bioinformatic searches were used to identify HER2 specific nucleic acid sequences around the HER2 target region. The selected genomic target nucleic acid sequence is separated into consecutive non-overlapping 80 nt segments. One thousand one hundred and ninety-six (1196) ~80mer oligonucleotides were synthesized each carrying 5 DNP haptens on an abasic phosphoramidite spaced 20 nt apart. A representative structure for these oligonucleotides is shown in FIG. 1(A) - (B). The bolded portion of FIG. 1(A), also SEQ. ID NO: 1, is shown in more detail in FIG. 1(B). The oligonucleotides were affinity purified and analyzed by mass spectrometry and gel electrophoresis. HER2 oligonucleotide probe was bulked in a formamide-based buffer without human blocking DNA. In the initial screening process, the number of oligonucleotides, the number and spacing of DNP haptens were functionally tested in the formamide-based buffer without human blocking DNA for sensitivity and specificity to HER2 gene.

[0153] The double-stranded HER2 probe (HER2 ds probe) was DNP-labeled with the same HER2 DNA template in the INFORM HER2 DUAL ISH DNA Probe. HER2 ds probe was formulated with 4mg/ml human blocking DNA in the formamide-based buffer. HER2 ds probe was only used in the single ISH assay.

[0154] The above-mentioned commercial product INFORM HER2 DUAL ISH DNA contains a dispenser containing 0.75ug/ml of a DIG-labeled Chr17 probe cocktailed with the DNP-labeled HER2.

[0155] A single strand oligonucleotide Chr17 probe (Chr17 oligonucleotide probe) was made with a pool of 14 oligonucleotides with lengths from 58bp to 87bp. Each oligonucleotide was labeled with two DIG hapten molecules on a non-binding tail having the sequence TATTTTTATTTT at its 5' end (*See* FIG. 2(A) - (C), wherein FIG. 2(A) shows an exemplary Chr17 probe sequence (SEQ. ID. NO: 2) including the 5' tail and FIG. 2(B) shows a more detailed structure of the aminoC6+Dig bolded region of FIG. 2(A) and FIG. 2(B) shows a more detailed structure of the Am~Uni+Dig bolded region. These oligonucleotides were PAGE purified and analyzed with mass spectrometry. The Chr17 oligonucleotide probe was formulated in a formamide-based buffer without human blocking DNA. In the initial screening process, a total of 28 oligonucleotides were tested for specificity to the chromosome 17 centromere. They were individually formulated in the formamide-based buffer without human blocking DNA for this initial screening as a pool for testing as a DISH assay. HER2 oligonucleotide probe (15µg/ml) and CHR17 oligonucleotide probe (0.5µg/ml) were formulated in the formamide-based buffer without human blocking DNA. In illustrative embodiments, the Chr 17 probe comprises one or more of the sequences listed in TABLE 3.

TABLE 3. Chromosome 17 probe sequences

Oligo name	Sequences	Length
CHR17_M1.1 SEQ ID.NO: 3	AATTCGTTGGAAACGGGATAATTTCAGCTGACTAAACAGAAGCA GTCTCAGAATCTTCTTTGTGATGTTTGCATTCAAA	79

Oligo name	Sequences	Length
CHR17_M2.1 SEQ ID.NO: 4	CTTCGTTCGAAACGGGTATATCTTCACATGCCATCTAGACAGAA GCATCCTCAGAAGCTTCTCTGTGATGACTGCATTC	79
CHR17_M2.2 SEQ ID.NO: 5	TGAACTCTCCTTTTGAGAGCGCAGTTTTGAAACTCTCTTTCTGTGG CATCTGCAAGGGGACATGTAGACCTCTTTGAAG	79
CHR17_M3.1 SEQ ID.NO: 6	TTTCGTTGGAAACGGAATCATCTTCACATAAAAACTACACAGAT GCATTCTCAGGAACTTTTTGGTGATGTTTGTATTC	79
CHR17_M5.1 SEQ ID.NO: 7	CCTATGGTAGTAAAGGGAATAGCTTCATAGAAAAACTAGACAGA AGCATTCTCAGAAAATACTTTGTGATGATTGAGTTTAAC	83
CHR17_M5.2 SEQ ID.NO: 8	CACAGAGCTGAACATTCCTTTGGATGGAGCAGGTTTGAGACACT CTTTTTGTACAATCTACAAGTGGATATTTGGACCTCTCTGAGG	87
CHR17_M8.2 SEQ ID.NO: 9	GTTTCACATTGCTTTTCATAGAGTAGTTCTGAAACATGCTTTTCGT AGTGTCTACAAGTGGACATTTGGAG	71
CHR17_M9.1 SEQ ID. NO: 10	CCTGTGGTGGAAAACGAATTATCGTCACGTAAAAACTAGAGAGA AGCATTGTCAGAAA	58
CHR17_M9.2 SEQ ID. NO: 11	TGCATTCAACTCACAGAGTTGAAGGTTCCTTTTCAAAGAGCAGTT TCCAATCACTCTTTGTGTGG	65
CHR17_M11.2 SEQ ID. NO: 12	CATTCCCTTTGACAGAGCAGTTTGGAAACTCTCTTTGTGTAGAAT CTGCAAGTGGAGATATGGACCGCTTT	71
CHR17_M12.1 SEQ ID. NO: 13	CCTATGGTAGTAAAGGAAATAGCTTCATATAAAAGCTAGACAGT AGCATTCACAGAAAACTCTTGGTGACGACTGAGTTT	80
CHR17_M13.1 SEQ ID. NO: 14	ATTTCGTTGGAAACGGGATAAACCGCACAGAACTAAACAGAAG CATTCTCAGAACCTTCTTCGTGATGTTTGCATTCAAC	80
CHR17_M16.1 SEQ ID. NO: 15	CGTAGTAAAGGAAATAACTTCCTATAAAAAGAAGACAGAAGCTT TCTCAGAAAATTCTTTGGGATGATTGAGTTGAACTC	80
CHR17_M16.2	ACACAGCTGACCATTCCTTCCGATCTAGCACTTTAGAAAGACAC	

Oligo name	Sequences	Length
SEQ ID. NO: 16	ACAGAGCTGAGCATTCCTTGCGATGTAGCAGTTTAGAAACACAC TTTCTGCAGAATCTGCAATTGCATATTTGGACCTT	80

C. Automated bright-field in situ hybridization for interphase slides

[0156] The BenchMark ULTRA automated slide processing system (Ventana Medical Systems, Inc., Tucson, Arizona) was used for the discovering and performance evaluation of the single strand oligonucleotide HER2 and/or CHR17 single and dual ISH assays for HER2 and CHR17 DNA targets. The FDA-approved INFORM HER2 DUAL ISH DNA Probe Assay protocol was used for tissue staining. A modification was introduced for shorter hybridization times (*i.e.* 16min, 32min and 1hr). In certain testing scenarios, the single strand oligonucleotide HER2 and/or Chr17 probes were used in the over-labeled INFORM HER2/Chr17 probe dispenser. INFORM HER2 DUAL ISH DNA Probe Assay reagents include dinitrophenyl (DNP)-labeled HER2 and digoxigenin-labeled (DIG) Chr17 probe cocktails, the ultraView SISH and ultraView Alkaline Phosphatase Red ISH detection kits (Ventana Medical Systems, Inc.). The slides were deparaffinized at 69°C, followed by incubation with pH 6 citrate buffer at 82°C and by digestion by ISH Protease 3 for 20 minutes. The probe(s) were first denatured for 8 minutes at 80°C, then hybridized for a set-up time (6 hours is the default for the FDA-approved protocol) at 44°C, followed by 3 stringency washes with pH 6.0 citrate buffer at 72°C. After the application of a horseradish peroxidase-labeled rabbit anti-DNP antibody linker, the specific hybridization of the DNP-linked HER2 probe to its target was visualized by an insoluble precipitate of silver chromogen. After the application of an alkaline phosphatase-labeled mouse anti-digoxigenin antibody linker, the visualization of digoxigenin-linked Chr17 probe was detected by the soluble precipitate of the alkaline phosphatase-based Fast Red chromogenic system. For visualizing the complete morphology of the tissue, the slides were counterstained with hematoxylin for 4 minutes and post-counterstained with bluing reagent for 4 minutes.

D. Automated bright-field chromosome metaphase spread ISH staining:

[0157] Metaphase chromosomes (CGH Metaphase Target Slides, Abbott Molecular) were UV crosslinked on Stratalinker 2400 (Stratagene Model # C00518) at energy level 200 mJ. They were then treated with 1% trypsin (Sigma cat#T1426) at room temperature for 5s. The slides were then processed for ISH staining under the same conditions as described above except skipping steps for baking, deparaffin, cell conditioning and counterstaining. After the staining is completed on the instrument, slides were stained with 4% Giemsa (Gibco, cat#10092-03) diluted in Gurr buffer (Gibco, cat#10582-013) at room temperature for 5 min, and the staining was visualized with a regular light microscope.

E Analytical Slide Scoring Criteria:

[0158] A board-certified pathologist (P.B.) with experience on interpreting HER2/Chr17 DISH stained slides reviewed and scored the slides. Each slide was scored for signal intensity and background. The analytical slide scoring criteria (TABLE 1) describe the "Acceptable" or "Not Acceptable" staining. The "Acceptable" or "Not Acceptable" criteria are corresponding to the capability whether the HER2 or the Chr17 pairs of signals are enumerable in 20 cells on a slide. The scoring criteria were developed and used as a stringent analytical tool for the purpose of assay optimization.

F. Signal Enumeration of HER2 and Chr17

[0159] Once an adequate target area was identified, the reader recorded the scores for HER2 and Chr17 copy numbers that were present in 20 representative nuclei. If the resulting HER2/Chr17 ratio falls within 1.8-2.2, the reader is recommended to score an additional 20 nuclei and the resulting ratio is calculated from the total 40 nuclei. HER2 gene status is reported as non-amplified (HER2/Chr17 < 2.0) or amplified (HER2/Chr17 > 2.0). Reference is made to *Interpretation Guide Ventana INFORM HER2 Dual ISH DNA Probe Cocktail Assay* for disclosure related to the assay.

G. HER2 oligonucleotide probe performance evaluation:

[0160] HER2 oligonucleotide probe hybridizes faster. A breast case with weak HER2 signal by INFORM HER2 DUAL ISH DNA Probe (FDA-approved protocol, 6hr hybridization) was selected. Duplicate slides were stained with HER2 oligonucleotide probe and HER2 ds probe (6.0ug/ml each) at shorter hybridization time (16, 32 and 60min). For 16min hybridization, HER2 oligonucleotide probe staining shows HER2 signals intensity 1.0 & 1.5 (FIG. 3(A) and (C)) while HER2 ds probe staining intensities are 0.5 & 0.5 (FIG. 3(B) and (D)). For 32min hybridization, HER2 oligonucleotide probe staining shows HER2 signals intensity 1.5 & 1.5 while HER2 ds probe staining intensity is 1.0 & 1.5 (FIG. 3(A)). For 60min hybridization, HER2 oligonucleotide probe staining shows HER2 signals intensity 2.0 & 2.0 while HER2 ds probe staining intensities are 2.0 & 1.5 (FIG. 3(A)).

[0161] For 16min hybridization, HER2 oligonucleotide probe staining shows HER2 signals coverage 40% & 30% (FIG. 3(B) and (C)), while HER2 ds probe staining signal coverage is 5% & 20% (FIG. 3(B) and (D)). For 32min hybridization, HER2 oligonucleotide probe staining shows HER2 signal coverage 50% & 50%, while HER2 ds probe staining signal coverage is 30% & 25% (FIG 3(B)). For 60min hybridization, HER2 oligonucleotide probe staining shows HER2 signal coverage 55% & 60%, while HER2 ds probe staining signal coverage is 50% & 50% (FIG. 3(B)).

[0162] HER2 oligonucleotide probe did not show background signals on all the tested hybridization time points, while HER2 ds probe staining has mild background (0.75 and 0.25 at 32min, and 0.25 and 0 at 60min).

[0163] For 2hr hybridization, INFORM HER2 DUAL ISH DNA Probe staining shows HER2 signals intensity 2 & 2.5 and coverage of 60% &65%. For 6hr hybridization, INFORM HER2 DUAL ISH DNA

Probe staining shows HER2 signals intensity 2 & 2.5 and coverage of 65% & 65%. No background was observed on these slides.

[0164] The above data suggest HER2 oligonucleotide hybridizes faster than HER2 ds probe. Higher signal intensity and better coverage was demonstrated at the earlier time points of hybridization process.

[0165] HER2 oligonucleotide probe staining exhibited favorable staining when comparing to HER2 ds probe at the same concentrations (3, 6, 9, and 12µg/ml), hybridization times (1&2 hrs) and stringency wash temperatures (68, 70 and 72°C). 6µg/ml of HER2 oligonucleotide probe could achieve equivalent or better staining as 12µg/ml of HER2 ds probe.

[0166] HER2 oligonucleotide probe staining (12.0µg/ml) generates regular shape signals with uniformed sizes (FIG. 4(A)), while HER2 ds probe staining (12.0µg/ml) has irregular signal shape with different sizes (FIG. 4(B)). HER2 oligonucleotide probe generates minimal background signal (FIG. 4(A)), while HER2 ds probe staining has some nuclear dusting background (FIG. 4(B)). Since HER2 oligonucleotide probe staining demonstrated minimal background signal when used at normal concentration ranges, we challenged it with extremely high concentration (24µg/ml). HER2 oligonucleotide probe staining revealed brownish background that surrounds nuclear boundary, however, this background pattern does not interfere with signal enumeration. HER2 ds probe staining at 24µg/ml exhibited nuclear dusting that may confuse weak specific signals from non-specific background signals.

[0167] HER2 oligonucleotide probe with 1hr hybridization staining is robust enough to reach the performance level of INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. In particular, 109 breast tissues were selected for HER2 oligonucleotide probe performance evaluation. These samples were first stained with INFORM HER2 DUAL ISH DNA Probe (FDA-approved protocol, 6hr hybridization), and demonstrated adequate or "boarder-line" staining intensity (2 is cut-off for acceptable, 1.5 is boarder-line). This pre-screening helped eliminate poor-quality tissues due to pre-analytical conditions. 79 tissues (72.5%) were considered passed by INFORM HER2 DUAL ISH DNA Probe. *See* TABLE 4.

TABLE 4.

109 breast cases with minimally adequate tissue quality	HER2 PMA 6hr hyb	HER2 ds 1hr hyb	HER2 oligo 1hr hyb
# of passed tissue (%)	79 (72.5%)	32 (29.3%)	94 (86.2%)

[0168] HER2 oligonucleotide probe (12µg/ml) 1hr hybridization staining had 94 (86.2%) tissues passed, while HER2 ds probe 1hr hybridization had 32 (29.3%) tissues passed (TABLE 4). The data suggest HER2 oligonucleotide probe staining is robust enough to reach the level of performance of INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. Among the 79 tissues with ≥2 intensity by INFORM HER2 DUAL ISH DNA Probe (FIG. 5(A)), HER2 oligonucleotide probe achieved comparable performance (77 passed). Among the 30 tissues with 1.5 intensity by INFORM HER2 DUAL ISH DNA Probe (FIG. 5(B)), 17 tissues' staining was improved to intensity 2 (therefore passed).

H. Chromosome 17 oligonucleotide probe performance evaluation:

Selection of chromosome 17-specific oligonucleotides

[0169] Each of the 28 chromosome 17 oligonucleotides (1.0 µg/ml) was stained on 2 breast tissues at stringency wash temperature 70 and/or 72°C. Fourteen oligonucleotides were excluded due to the extra cross-reactive signals, *i.e.* M4.5 (2.0&2.5), M6.1 (0&0.25), M6.2 (0.5&2.5), M7.2 (1.0&1.5), M8.1 (2.0&2.0), M10.1 (1.5&2), M10.2 (2&2.5), M11.1 (0&2), M14.1 (0.75&1.0), M14.2 (1.5&1.5), M15.1 (0&0.25), and M15.2 (2.0&2.0) (FIG. 6(B)). FIG. 6(D) is an example of M11.2 stained slide, one to two specific chr17 signals with adequate intensity are present in each cell. FIG. 6(C) shows an example of M7.2 stained slide, extra faint cross-reactive signals were present in addition to the major chr17 specific signals in the cells. Two oligonucleotides were excluded for the extremely weak signals, *i.e.* M1.2 (0.25&0.25) and M2.2 (0&0.25) (FIG. 6(A)). A total of 14 oligonucleotides (M1.1, M2.1, M2.2, M3.1, M5.1, M5.2, M8.2, M9.1, M9.2, M11.2, M12.1, M13.1, M16.1, and M16.2) were chosen enter the pool of Chr17 oligonucleotide probe as listed herein.

[0170] Fifteen (15) breast tissues were selected for Chr17 oligonucleotide probe performance evaluation. The chromosome 17 staining was performed earlier with INFORM HER2 DUAL ISH DNA Probe (FDA-approved protocol, 6hr hybridization). Chromosome 17 signals in these samples (duplicate slides each) range from strong to weak intensities (0-3 scale). Chr17 oligonucleotide probe (0.5µg/ml) 1hr hybridization staining has comparable staining intensity (2.60±0.61 vs 2.54±0.84, $p>0.05$, FIG. 7(A)), coverage (70.50±14.46 vs 66.93±24.61, $p>0.05$, Figure 5B) and background (0.04±0.16 vs 0.02±0.07, $p>0.05$, FIG. 7(C)) to those by Chr17 PMA probe. All non-acceptable chr17 staining are due to inadequate chr17 signal intensity, among which 2 failed by Chr17 oligonucleotide probe and 5 by Chr17 PMA probe (FIG. 7(D)). The data suggest Chr17 oligonucleotide probe 1hr hybridization staining is robust enough to reach the level of performance of INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization.

Analytical characterization of HER2/CHR17 Oligonucleotide Probe DISH assay

[0171] Analytical specificity of HER2/CHR17 Oligonucleotide Probe DISH on chromosomal metaphase spreads was tested. HER2 oligonucleotide probe (black signal) and CHR17 oligonucleotide probe (red signal) were localized to the same chromosome. No cross-hybridization of either HER2 probe or CHR17 probe to other chromosomes was observed (FIG. 8).

[0172] Functional test on the minimally required number of HER2 oligonucleotides was tested. Forty-eight (48), 72 and 100% of the total number (1196) HER2 oligonucleotides were functionally tested on 30 slides from 5 breast cases. All slides passed for HER2 staining (with the criteria Intensity ≥2). 48% of 1196 HER2 oligonucleotides had HER2 intensity 2.00±0,72% had HER2 intensity 2.45± 0.16, and 100% had HER2 intensity 2.75 ±0.35. 100% (1196 oligonucleotides) had the most robust HER2 staining comparing to those by 48% and 72% ($p<0.05$). 48% of 1196 HER2 oligonucleotides had HER2 coverage 69.00 ± 4.60, 72% had HER2 coverage 73.50 ± 2.42, and

100% had HER2 coverage 77.50 ±5.89. 100% (1196 oligonucleotides) had significantly higher HER2 staining coverage than that by 48% ($p<0.05$) (FIG. 9).

[0173] Functional test of the time course (1, 2 and 6hr) of the full-set (1196) Her2 oligonucleotides was tested. The full-set (1196) Her2 oligonucleotides were tested for 1, 2 and 6hr hybridization on 16 slides from 4 breast cases. HER2 oligonucleotides with 1hr hybridization staining achieved comparable staining performance to INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. We did not find consistent linkage between the longer hybridization times (*e.g.* 2 and 6hr) and improved staining intensity (FIG. 10).

[0174] An equivalency study of HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization on individual breast tissues was performed to compare staining adequacy compared to INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. Eighty-nine (89) breast tissues were selected for HER2/CHR17 Oligonucleotide Probe DISH performance evaluation. Similar to above, these samples demonstrated adequate or "boarder-line" staining intensity, *i.e.* HER2 signal intensity ≥ 1.5, CHR17 signal intensity ≥ 1.5 on at least one slide stained by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. This pre-screening helped eliminate poor-quality tissues due to pre-analytical conditions. 128 slides (128/146, 85.5%) were considered "pass" for HER2 staining by INFORM HER2 DUAL ISH DNA Probe with 1hr hybridization, while 156 slides (156/174, 87.67%) were considered pass for HER2 staining by HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization (p=0.578). 103 slides (103/149, 69.13%) were considered pass for CHR17 staining by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization, while 129 slides (129/175, 73.71%) were considered pass for CHR17 staining by HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization (p=0.363). No significant difference was found for HER2 and CHR17 staining between the two assays. While no severe speckling or slide drying artifact was found for HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization, 6 slides stained by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization failed for evaluation due to severe speckling background, and 5 slides stained by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization failed for slide drying (11/175, 6.3%). See TABLE 5.

TABLE 5.

	HER2 signal			
	1st-Pass	Fail	Pass rate	Significance
HER2/Chr17 oligonucleotide probe DISH	156	18	87.67%	p=0.578
INFORM HER2 DUAL ISH DNA Probe	128	18	85.50%	
	CHR17 signal			
	1st-Pass	Fail	Pass rate	Significance
HER2/Chrl7 oligonucleotide probe DISH	129	46	73.71%	p=0.363
INFORM HER2 DUAL ISH DNA Probe	103	46	69.13%	

Artifacts	INFORM HER2 DUAL ISH DNA Probe	HER2/Chrl7 oligonucleotide probe DISH
Speckling (Background 2, failure)	6	0
Slide (failure)	5	0

[0175] FIG. 11(A) is an example of staining on Case#709. HER2/CHR17 Oligonucleotide Probe ISH 1hr hybridization staining had HER2 intensity 2.5, coverage 70%, background 0; Chr17 intensity 2.5, coverage 70%, and background 0; while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining had HER2 intensity 1, coverage 40%, background 0; Chr17: Intensity 1, coverage 35%, background 0. In the circled stromal areas, INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization had lack of staining; hence the HER2 and CHR17 staining intensity were assigned to 1. The data suggest HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining performance is comparable to that of INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining has lower incidence of staining failure (*i.e.* severe speckling and slide drying) than that by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization.

[0176] Concordance of HER2 gene status between HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining

[0177] Sixty-three (63) cases with paired slides of ≥2 intensity for both HER2 and CHR17 by HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization were selected for signal enumeration. Fifty (50) cases were diagnosed as HER2 non-amplified by both HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining. Twelve (12) cases were diagnosed as HER2 amplified by both HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining. One case (ILS32554) was diagnosed as HER2 amplified by HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining (HER2/Chr17 ratio: 2.08), while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining is non-amplified (HER2/Chr17 ratio: 1.92). The Percent Positive Agreement (PPA) is 100% (95% score CI: 77.1-100%), and the Percent Negative Agreement (PNA) is 98.04% (95% score CI: 92.7-98.0%). The percentage of Coefficient of Variation (%CV) of non-clustered signal counts (for HER2 and CHR17) from the paired slides is 5.66±4.84 (<20% as acceptable). *See* TABLE 6.

TABLE 6

	INFORM HER2 DUAL ISH DNA Probe		
HER2/CHR17 oligonucleotide probe DISH	**Amplified**	**Non-Amplified**	**Total**
Amplified	12	1*	12
Non-Amplified	0	50	51
Total	12	51	63

*ILS32554: PMA 1.92, Oligonucleotide 2.08		
	n/N	**% (95% Score CI)**
Percent Positive Agreement (PPA)	12/12	100 (77.1-100)
Percent Negative Agreement (PNA)	50/51	98.04 (92.7-98.0)

[0178] FIG. 11(B) is an example of staining on Case#731. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining had HER2 intensity 3, coverage 80%, background 0; Chr17: Intensity 2.5, coverage 75%, background 0; HER2 counts: 46, Chr17 counts: 34, ratio: 1.35; while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining had HER2 intensity 3, coverage 80%, background 0.5; CHR17 intensity 3, coverage 80%, background 0. Both stains generated similar HER2 and CHR17 signal counts, and hence similar HER2/CHR17 ratio. Silver dusting background was observed on INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization stains. The data suggest HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining have a high concordance on the diagnoses of HER2 gene status.

[0179] An evaluation of the two assays' robustness on a cohort of tissue microarray (TMA) that was not prescreened by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization was completed. Ninety-five (95) breast tissue cores in a TMA slide were stained with HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization for the evaluation of assay robustness on these tissues without the information of pre-analytical conditions and tissue quality. This study was designed to assess the assay robustness as archived TMA tissues are generally considered difficult specimens for ISH assay. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization had 73 cores (76.8%) stained HER2 intensity 2 and above, while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization had 57 cores (60.0%) stained HER2 intensity 2 and above. The difference between the two assays on HER2 intensity reaches close to 90% CI significance (p=0.011). HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization had 53 cores (55.8%) stained CHR17 intensity 2 and above, while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization had 35 cores (36.80%) stained CHR17 intensity 2 and above. The difference between the two assays on CHR17 intensity reaches close to 90% CI significance (p=0.012). HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization stained slides had 1.89±0.76 on HER2 intensity, while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization stained slides 1.58±0.76 on HER2 intensity (p=0.005). HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization stained slides had 1.49±0.83 on CHR17 intensity, while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization stained slides 1.04±0.87 on CHR17 intensity (p=0.000). The background for both HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization (0.11±0.18) and INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization (0.04±0.11) are very low from the acceptable level (<2). *See* TABLE 7.

TABLE 7.

The number of slides at given signal intensity level						
Intensity level	INFORM HER2 DUAL ISH DNA Probe (6hr hyb)	HER2/CHR17 oligonucleotide probe DISH (1hr hyb)	Significance	INFORM HER2 DUAL ISH DNA Probe (6hr hyb)	HER2/CHR17 oligonucleotide probe DISH (1hr hyb)	Significance
	HER2 signal	HER2 signal		CHR17 signal	CHR17 signal	
3	0	5		0	0	
2.5	10	28		4	16	
2	47	40		29	36	
1.5	16	2		11	9	
1	3	10		4	7	
0.5	8	4		21	18	
0	11	6		26	9	
Total tissue cores	95	95		95	95	
Percentage passed tissue cores	60.0% (57/95)	76.8% (73/95)	p=0.011	35/95 (36.8%)	53/95 (55.8%)	p=0.012

HER2 and CHR17 staining scores			
	INFORM HER2 DUAL ISH DNA Probe (6hr hyb)	HER2/CHR17 oligonucleotide probe DISH (1hr hyb)	Significance
HER2 Intensity	1.58±0.76	1.89±0.76	p=0.005
HER2 Background	0.04±0.11	0.11±0.18	p=0.001
CHR17 Intensity	1.04±0.87	1.49±0.83	p=0.000
CHR17 Background	0	0	na

[0180] The data suggest HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization has more robust staining on difficult tissues than INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization.

[0181] In addition to testing on breast tissues, the feasibility of HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization was also demonstrated on lung (FIG. 12(A) showing the single strand probe and 12(B) showing the double strand probe) and gastric tissues (FIG. 13(A) showing

the single strand probe and 13(B) showing the double strand probe). We further tested HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization on duplicate slides of 10 lung tissues and 10 gastric tissues. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization has 65% pass for HER2 and 50% pass for CHR17 (based on criteria intensity ≥2), similar to 55% pass for HER2 and 50% pass for CHR17 by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization (p=0.516 for HER2 and p=1.000 for CHR17). FIG. 12(A) - (B) is an example of staining on Case# F101411A1. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining had HER2 intensity 3, coverage 80%, background 0; Chr17: Intensity 3.0, coverage 80%, background 0; while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining had HER2 intensity 3, coverage 80%, background 0.5; CHR17 intensity 3, coverage 80%, background 0. Some silver background was observed on the tissue stained by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization.

[0182] We chose 10 gastric tissue cases that were adequately stained by INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization. Duplicate slides for each case were stained with HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization. 18 slides stained by HER2/CHR17 Oligonucleotide Probe DISH passed (based on criteria intensity ≥2). For 2 cases, one of the duplicate slides had inadequate staining by HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization. FIG. 13 (A) - (B) is an example of staining on Case# I-5189-C8a. HER2/CHR17 Oligonucleotide Probe DISH with 1hr hybridization staining had HER2 intensity 3, coverage 80%, background 0; Chr17: Intensity 3.0, coverage 80%, background 0; while INFORM HER2 DUAL ISH DNA Probe with 6hr hybridization staining had HER2 intensity 2.5, coverage 70%, background 0.5; CHR17 intensity 2.5, coverage 75%, background 0.

EXAMPLE 2

[0183] Example 2 compares the p17H8 plasmid and the 42mer CHR17 oligonucleotide probe (and determines compatibility of the 42mer CHR17 oligonucleotide probe with the HER2 oligonucleotide probe).

[0184] p17H8 plasmid (PMA): The p17H8 plasmid probe contains this entire sequence. There are 16 repeats of the 166bp sequence. This probe requires human placenta DNA because it gives weak signal from cross-hybridization to other chromosomes.

[0185] CHR17 (42mer) oligo: The 42mer CHR17 oligonucleotide probe (Ventana P/N: 90682, 10760, 95221) has good specificity for CHR17, does not require human placenta DNA, but the optimal hybridization conditions are different from the HER2 probe2 (INFORM FDA-approved product, made from PCR products). Its compatibility with the HER2 oligonucleotide probe is evaluated here.

[0186] Fixed assay conditions: StdCC2, P3 20min, Denaturation 8min, silver and red detection 8min, H&E 8min.

[0187] Conditions to be tested:

1. (1) Chr17 Oligonucleotide (42mer) concentrations: 0.35ug/ml, 0.5ug/ml, 0.75ug/ml, 1.5ug/ml and 3.0ug/ml.
2. (2) Hybridization temperatures: 42°C, 44°C, and 46°C.
3. (3) Hybridization time: 1hr, 2hrs, and 6 hrs.
4. (4) Stringency wash temperatures: 54°C, 59°C, and 65°C.
5. (5) Formamide concentration in the hybridization buffer: 22.8 % and 33.2%.

[0188] Results: FIG. 14(A) shows weak staining of the 42mer CHR17 oligonucleotide probe (Conditions were as follows: Chr17 Oligonucleotide (42mer) 0.75ug/ml, 46°C and 6hrs Hyb (hybridization), 59°C stringency wash, formamide concentration: 33.2%). FIG. 14(B) shows that Chr17 (42mer) staining is weaker than PMA at 33.2% formamide. Increasing the concentration and hybridization time did not increase the signal. (Conditions included a 59°C stringency wash, 33.2% formamide). FIG. 14(C) shows that 22.8% formamide gave a better CHR17 signal, but it was still weaker than PMA. Increasing the concentration and hybridization time did not increase the signal intensity. No background observed with 59°C stringency wash. However, 22.8% formamide is not optimal for HER2 Oligonucleotide ISH (not compatible). FIG. 14(D) shows that the stringency wash temperature for CHR17 oligonucleotide (42mer) is not compatible with the HER2 oligonucleotide (68-72°C); a 65°C stringency temperature reduced CHR17 signals.

[0189] In summary, the 42mer CHR17 Oligonucleotide probe generates specific signal, and 0.75 and 1.5ug/ml for 1hr offer the best staining. But, the staining is weaker than PMA control. Increasing the concentration and hybridization time did not improve signal intensity. The hybridization assay conditions for the 42mer CHR17 oligonucleotide probe are not compatible with that of HER2 oligonucleotide probe: the optimal range of stringency wash temperatures for HER2 oligonucleotide is 68-72°C; and the drop on staining intensity of CHR17 oligonucleotide (42mer) becomes obvious when the temp goes up to 65°C.

[0190] While a number of exemplary aspects and embodiments have been discussed above, those of skill in the art will recognize certain modifications, permutations, additions and sub-combinations thereof as being present in the disclosure.

TABLE 8. Sequences

SEQ. ID. NO: 1	TCTCGTCTCGGCCCCCGACCTGCGTCCTGGGCCCGCAGGGGAGTCCTGCC CCATGCTCCCGGGCGGGGCCGCCCTGTGCCCT
SEQ. ID. NO: 2	TATTTTTATTTTAATTCGTTGGAAACGGGATAATTTCAGCTGACTAAACAGA AGCAGTCTCAGAATCTTCTTTGTGATGTTTGCATTCAAA
SEQ ID. NO: 3 CHR17_M1.1	AATTCGTTGGAAACGGGATAATTTCAGCTGACTAAACAGAAGCAGTCTCAGA ATCTTCTTTGTGATGTTTGCATTCAAA
SEQ ID. NO: 4	CTTCGTTCGAAACGGGTATATCTTCACATGCCATCTAGACAGAAGCATCCTCA

CHP17_M2.1	GAAGCTTCTCTGTGATGACTGCATTC
SEQ ID. NO: 5 CHP17_M2.2	TGAACTCTCCTTTTGAGAGCGCAGTTTTGAAACTCTCTTTCTGTGGCATCTGCA AGGGGACATGTAGACCTCTTTGAAG
SEQ ID. NO: 6 CHP17_M3.1	TTTCGTTGGAAACGGAATCATCTTCACATAAAAACTACACAGATGCATTCTCA GGAACTTTTTGGTGATGTTTGTATTC
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REFERENCES CITED IN THE DESCRIPTION

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PATENTKRAV

1. System til *in situ*-påvisning af et kontrolområde af humant kromosom 17, hvilket system omfatter:

et sæt af to eller flere enkeltstrengede kontrolprober, der er specifikke for X særskilte monomerer af et alfasatellit-kontrolområde af humant kromosom 17, hvor X = 2-14, hver kontrolprobe er mærket med mindst ét første mærke, og hvor hver kontrolprobe omfatter:

- en sekvens udvalgt fra gruppen bestående af SEQ ID NO: 3-16, eller
- en sekvens udvalgt fra gruppen bestående af en trunkeret version af SEQ ID NO: 3-16, hvilken trunkeret version er mindst 40 sammenhængende bp af SEQ ID NO: 3-16, eller
- en sekvens udvalgt fra gruppen bestående af en sekvens, der har mindst 70 % sekvensidentitet med én af SEQ ID NO: 3-16, eller
- komplementer deraf.

2. System ifølge krav 1, hvor hver kontrolprobe omfatter:

- en sekvens udvalgt fra gruppen bestående af en trunkeret version af SEQ ID NO: 3-16, hvilken trunkeret version er mindst 40 sammenhængende bp af SEQ ID NO: 3-16, eller
- en sekvens udvalgt fra gruppen bestående af en sekvens, der har mindst 70 % sekvensidentitet med én af SEQ ID NO: 3-16 eller
- komplementer deraf.

3. System ifølge et hvilket som helst af kravene 1 eller 2, hvor hver kontrolprobe omfatter mellem 50 og 100 nukleotider.

4. System ifølge et hvilket som helst af kravene 1 til 3, hvor kontrolproberne er rettet mod mellem 2 og 14 særskilte dele inden for kontrolområdet.

5. System ifølge et hvilket som helst af kravene 1 til 4, hvor hver kontrolprobe er mærket med mindst 2, mindst 3, mindst 4 eller mindst 5 første mærker.

6. System ifølge et hvilket som helst af kravene 1 til 5, hvor i det mindste det ene første mærke omfatter et hapten.

7. System ifølge et hvilket som helst af kravene 1 til 6, der yderligere omfatter et målprobe, der er specifikt for et målområde af humant kromosom 17, hvor målproben er mærket med mindst ét andet mærke.

8. System ifølge krav 7, hvor målproben er specifik for et målområde nær eller

omkring HER2-genlocuset, eller hvor målproben er specifik for et område mellem nukleotiderne 35.027.979 og 35.355.516 af humant kromosom 17.

9. System ifølge et hvilket som helst af kravene 1 til 8, der yderligere omfatter et ISH-farvningsinstrument, hvilket instrument er konfigureret til at bringe kontrolproben i kontakt med en vævsprøve.

10. Kit omfattende en beholder, der indeholder et system ifølge et hvilket som helst af kravene 1 til 8.

11. Objektglas omfattende en flerhed af cellekerner, der er kromogent farvet for kromosom 17, hvor objektglasset frembringes ved hjælp af et system ifølge et hvilket som helst af kravene 1 til 9.

12. Fremgangsmåde til *in situ*-hybridisering af en vævsprøve, hvilken fremgangsmåde omfatter at bringe vævsprøven i kontakt med et system ifølge et hvilket som helst af kravene 1 til 9.

13. Fremgangsmåde til dobbelt lysfelt, *in situ*-hybridisering omfattende:

at bringe en vævsprøve i kontakt med et sæt af to eller flere enkeltstrengede kontrolprober, der er specifikke for X særskilte monomerer af et alfasatellit-kontrolområde af humant kromosom 17, hvor X = 2-14 samt hvor hver kontrolprobe mærket med mindst ét første mærke og omfatter:

- en sekvens udvalgt fra gruppen bestående af SEQ ID NO: 3-16, eller
- en sekvens udvalgt fra gruppen bestående af en trunkeret version af SEQ ID NO: 3-16, hvilken trunkeret version er mindst 40 sammenhængende bp af SEQ ID NO: 3-16, eller
- en sekvens udvalgt fra gruppen bestående af en sekvens, der har mindst 70 % sekvensidentitet med én af SEQ ID NO: 3-16, eller
- komplementer deraf,

at bringe vævsprøven i kontakt med en enkeltstrenget målprobe specifik for et målområde nær eller omkring HER2-genlocuset for humant kromosom 17, hvor målproben er mærket med mindst ét andet mærke,

at hybridisere proberne under betingelser i et tidsrum på mindre end omkring 3 timer,

at skylle prøven for at fjerne ubundet probe og

at farve prøven for at påvise hybridiserede prober.

14. Fremgangsmåde ifølge krav 13, hvor prøven farves med en første kromogen farve til påvisning af kontrolproberne og en anden, særskilt kromogen farve til påvisning af målproben

specifik for et målområde nær eller omkring HER2-genlocuset for humant kromosom 17.

15. Fremgangsmåde ifølge krav 13 eller 14, hvor proberne hybridiseres under betingelser i et tidsrum på mindre end omkring 2 timer, eventuelt hvor proberne hybridiseres under betingelser i et tidsrum på mindre end omkring 1 time.

16. Fremgangsmåde ifølge et hvilket som helst af kravene 13 til 15, hvor vævsprøven er en formalinfikseret paraffinindlejret (formalin-fixed paraffin-embedded FFPE) vævsprøve.

17. Fremgangsmåde til vurdering af et kromosom for HER2-genkopiantal, hvilken fremgangsmåde omfatter:

at opnå en vævsprøve, der har været igennem *in situ*-hybridisering ifølge et hvilket som helst af kravene 13 til 16, hvor der anvendes en kontrolprobe, der er specifik for humant kromosom 17, og en målprobe, der er specifik for HER2,

at identificere et område af neoplastiske cellekerner med højeste kopiantal,

at tælle tælbare signaler for HER2-signal i mindst 20 cellekerner og

at beregne forholdet mellem HER2-signal og kromosom 17-signal (HER2-/CHR17-forholdet).

18. Fremgangsmåde ifølge krav 17, hvor at hvis HER2-/CHR17-forholdet ligger mellem 1,8 og 2,2, tælles de tælbare signaler i yderligere 20 cellekerner, og HER2-/CHR17-forholdet beregnes ud fra samtlige 40 cellekerner.

19. Fremgangsmåde ifølge krav 17, hvor et HER2-/CHR17-forhold, der er mindre end 2,0, anses for at være ikke-amplificeret, og et HER2-/CHR17-forhold, der er mere end eller lig med 2,0, anses for at være amplificeret.

20. Fremgangsmåde ifølge et hvilket som helst af kravene 17 til 19, der yderligere omfatter beregning af det gennemsnitlige antal HER2-kopier per cellekerne.

DRAWINGS

5'-T[DNP]CTCGTCTCGGCCCCCGACCT
[DNP]GCGTCCTGGGCCCGCAGGGG[DNP]
AGTCCTGCCCCATGCTCC**CG[DNP]**
GGCGGGGCCGCCCTGTGCCC[DNP]T-3'

FIG. 1(A)

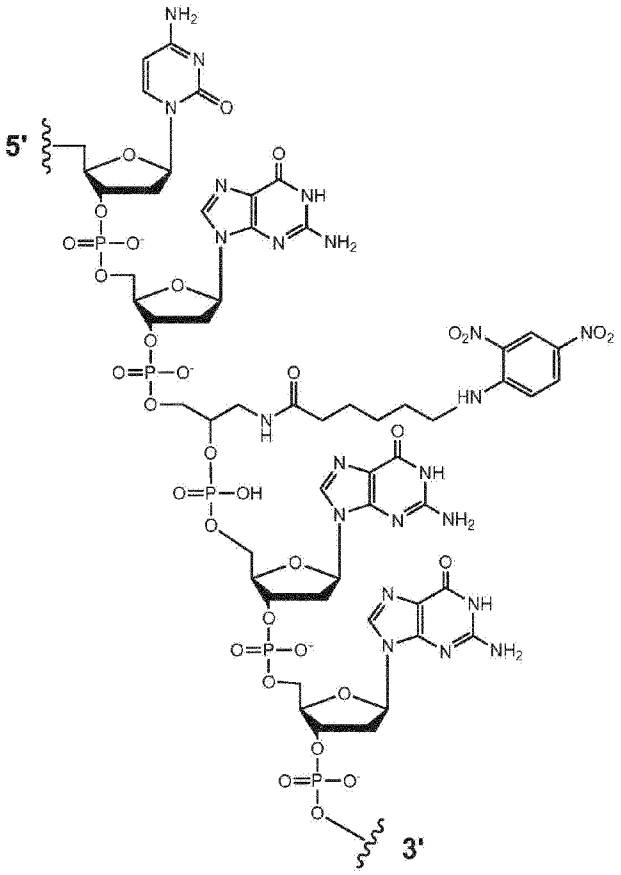

FIG. 1(B)

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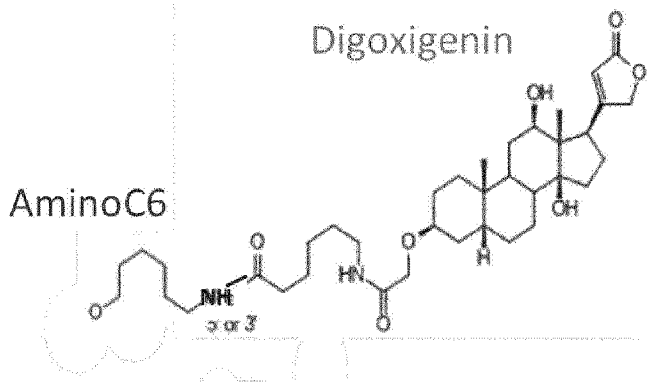
FIG. 2(A)



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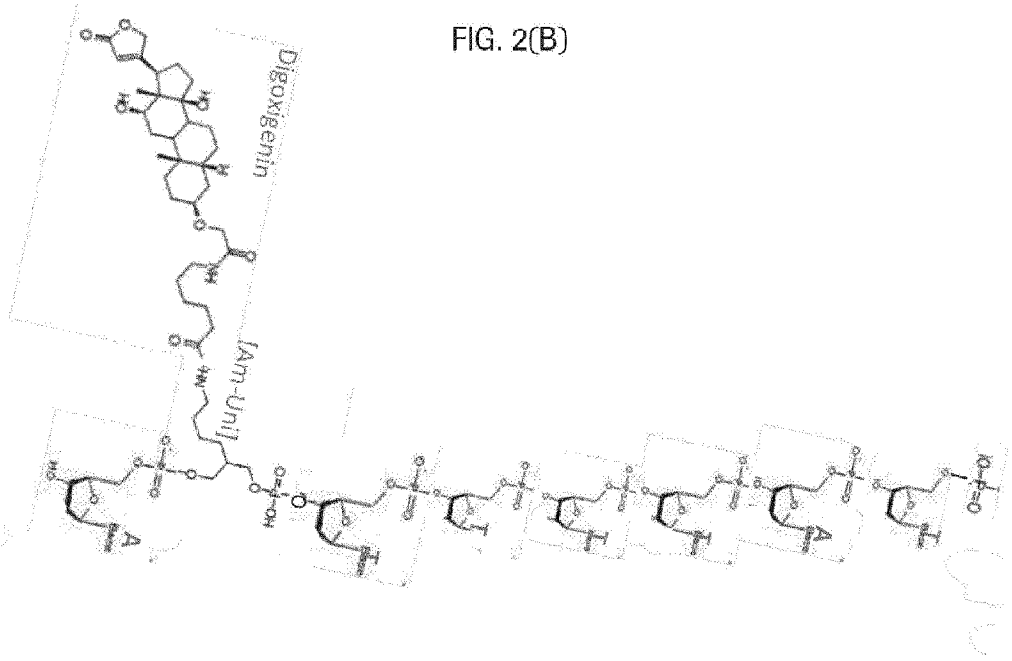

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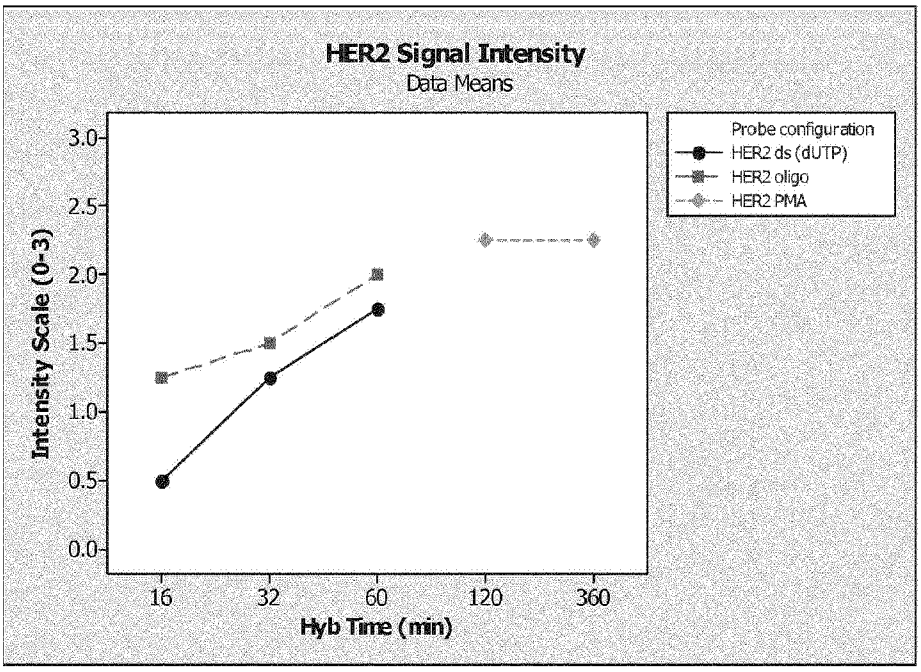
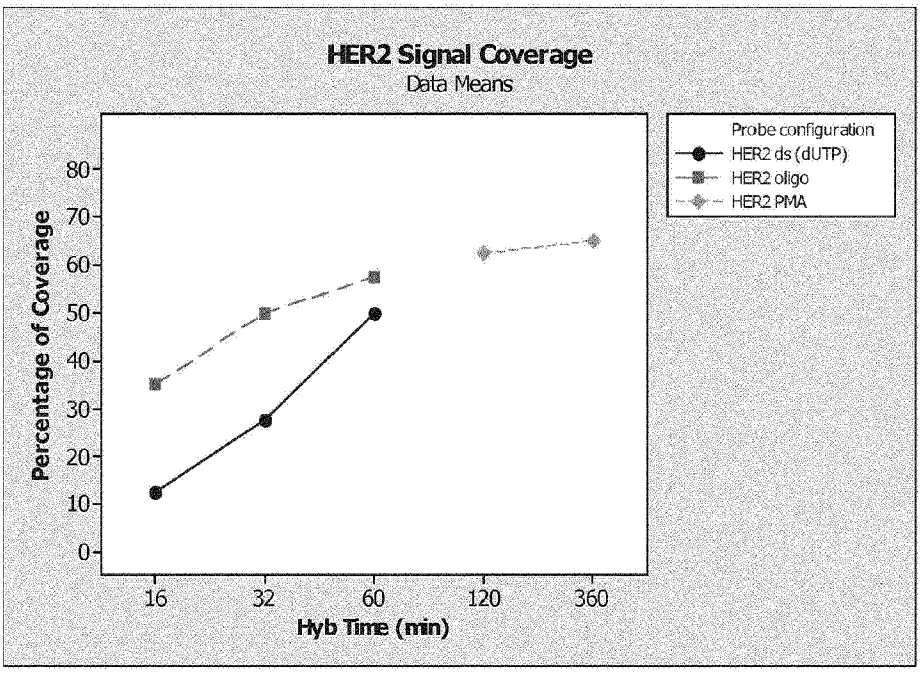

HER2 Signal Intensity
Data Means
Probe configuration
HER2 ds (dUTP)
HER2 oligo
HER2 PMA
Intensity Scale (0-3)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
16
32
60
120
360
Hyb Time (min)

FIG. 3(A)


HER2 Signal Coverage
Data Means
Probe configuration
HER2 ds (dUTP)
HER2 oligo
HER2 PMA
Percentage of Coverage
80
70
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50
40
30
20
10
0
16
32
60
120
360
Hyb Time (min)

FIG. 3(B)

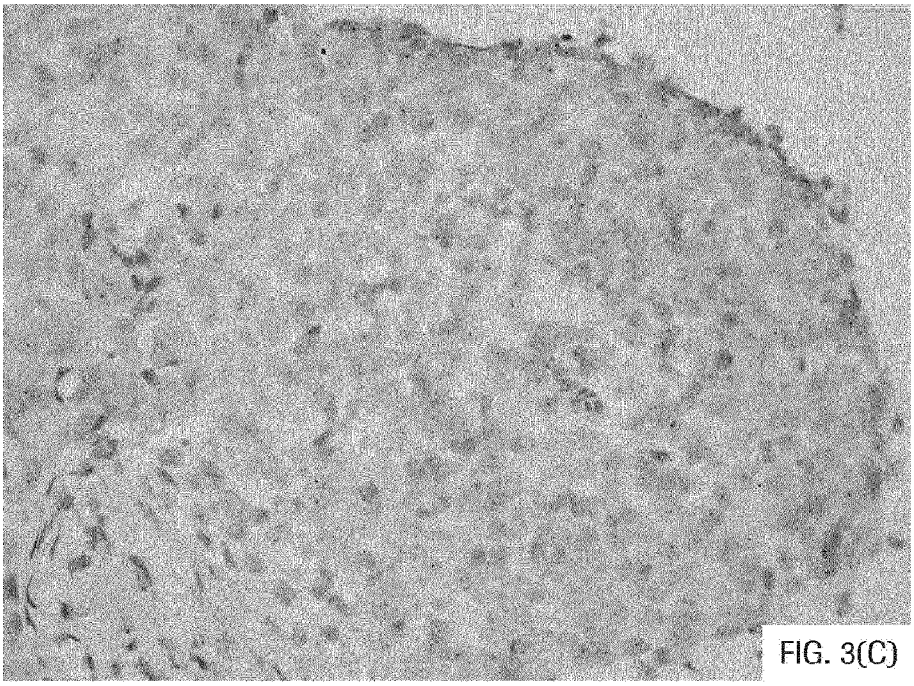

FIG. 3(C)

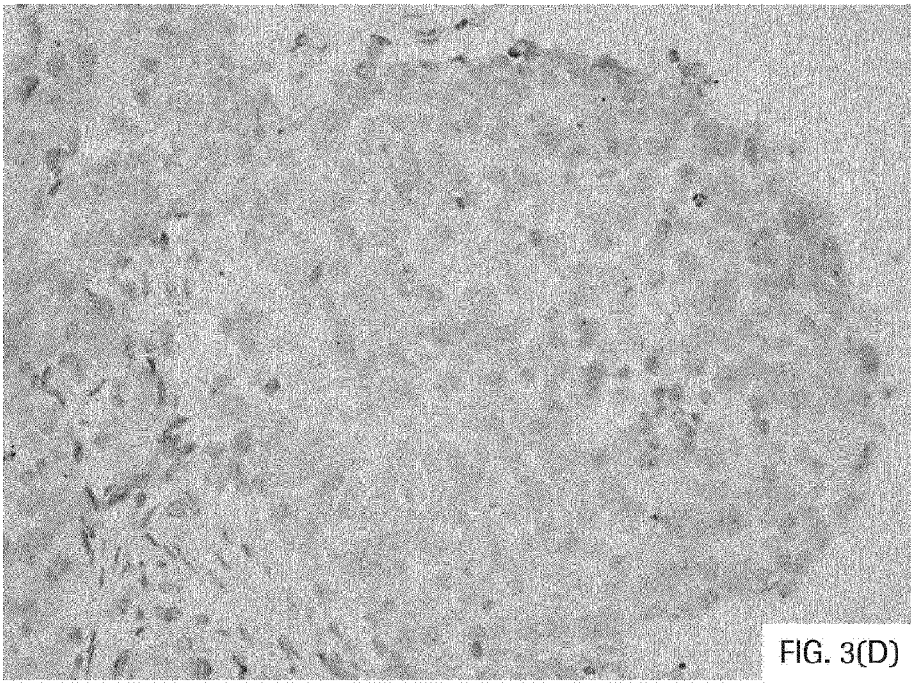

FIG. 3(D)

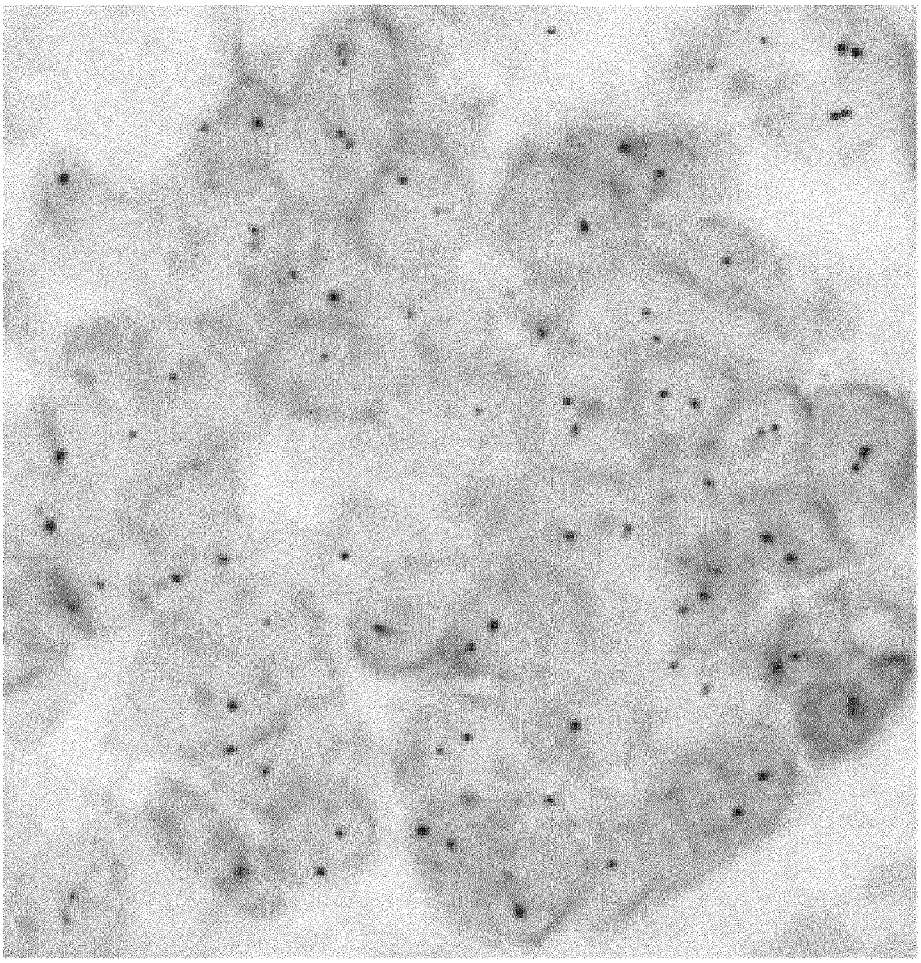

FIG. 4(A)

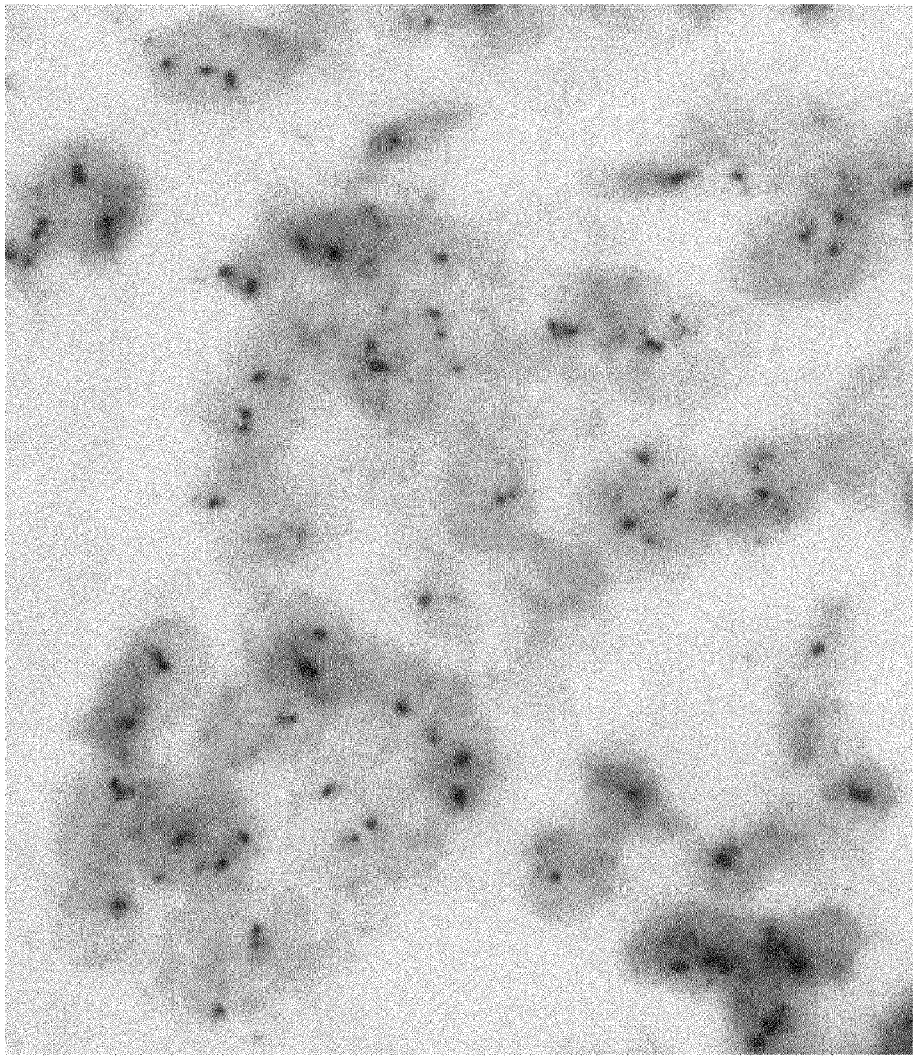

FIG. 4(B)

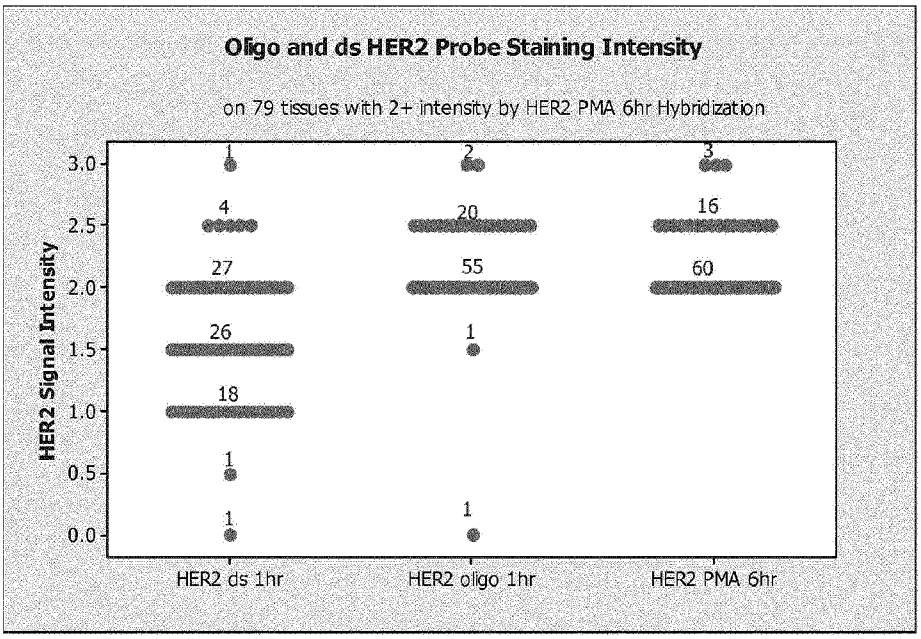
Oligo and ds HER2 Probe Staining Intensity
on 79 tissues with 2+ intensity by HER2 PMA 6hr Hybridization
HER2 Signal Intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
4
27
26
18
1
1
2
20
55
1
1
3
16
60
HER2 ds 1hr
HER2 oligo 1hr
HER2 PMA 6hr

FIG. 5(A)

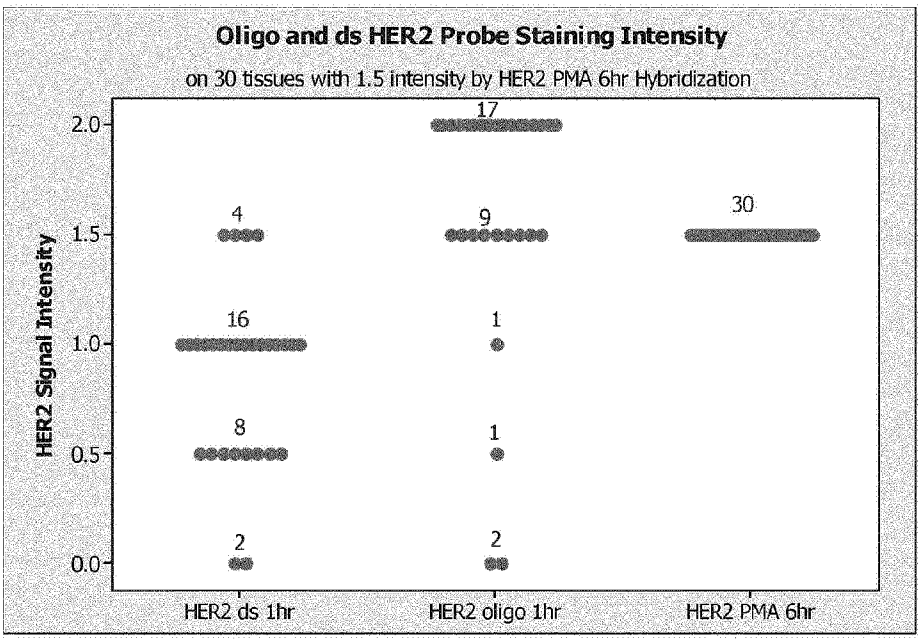
Oligo and ds HER2 Probe Staining Intensity
on 30 tissues with 1.5 intensity by HER2 PMA 6hr Hybridization
HER2 Signal Intensity
2.0
1.5
1.0
0.5
0.0
4
16
8
2
17
9
1
1
2
30
HER2 ds 1hr
HER2 oligo 1hr
HER2 PMA 6hr

FIG. 5(B)

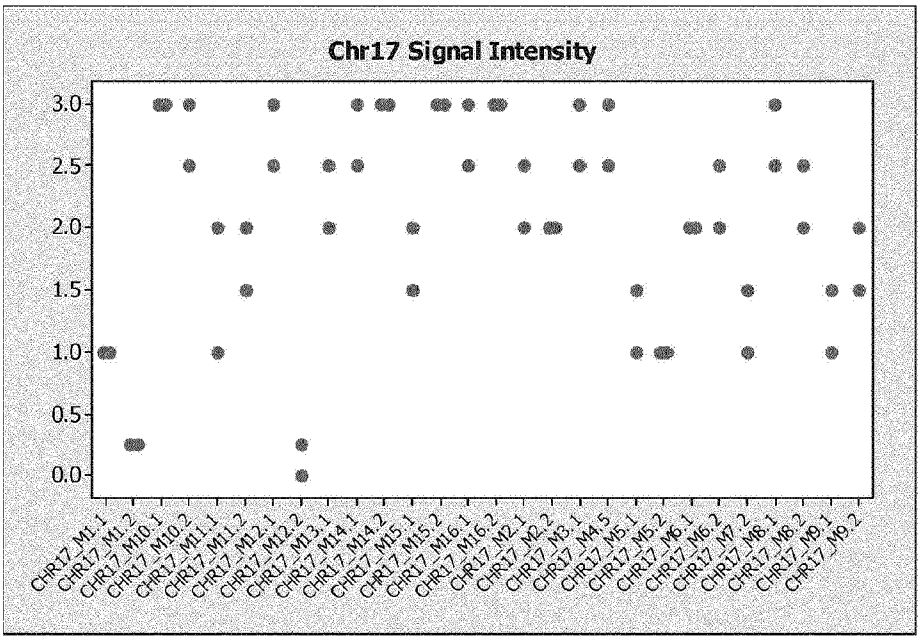
Chr17 Signal Intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CHR17_M1:1
CHR17_M1:2
CHR17_M10:1
CHR17_M10:2
CHR17_M11:1
CHR17_M11:2
CHR17_M12:1
CHR17_M12:2
CHR17_M13:1
CHR17_M14:1
CHR17_M14:2
CHR17_M15:1
CHR17_M15:2
CHR17_M16:1
CHR17_M16:2
CHR17_M2:1
CHR17_M2:2
CHR17_M3:1
CHR17_M4:5
CHR17_M5:1
CHR17_M5:2
CHR17_M6:1
CHR17_M6:2
CHR17_M7:2
CHR17_M8:1
CHR17_M8:2
CHR17_M9:1
CHR17_M9:2

FIG. 6(A)

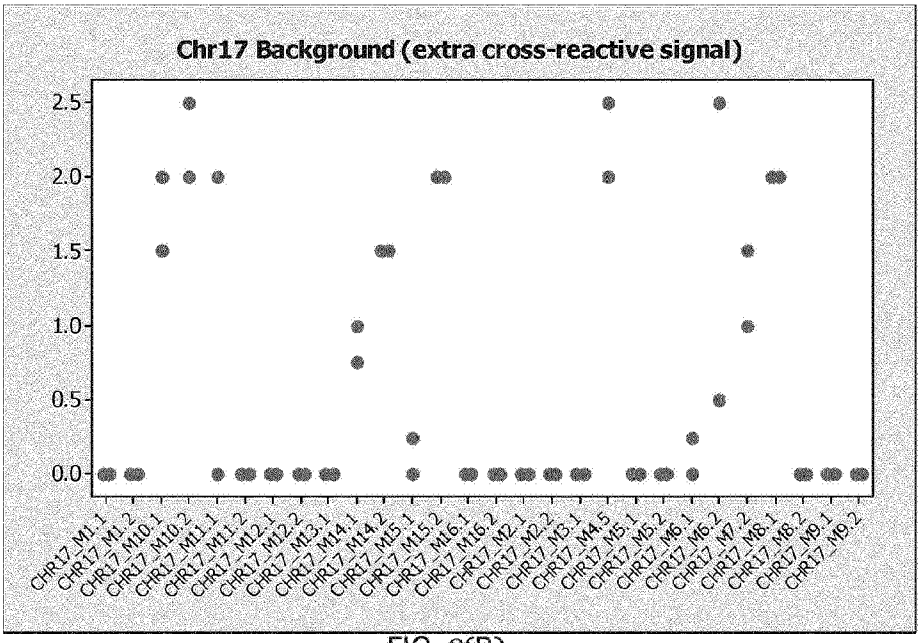
Chr17 Background (extra cross-reactive signal)
2.5
2.0
1.5
1.0
0.5
0.0
CHR17_M1:1
CHR17_M1:2
CHR17_M10:1
CHR17_M10:2
CHR17_M11:1
CHR17_M11:2
CHR17_M12:1
CHR17_M12:2
CHR17_M13:1
CHR17_M14:1
CHR17_M14:2
CHR17_M15:1
CHR17_M15:2
CHR17_M16:1
CHR17_M16:2
CHR17_M2:1
CHR17_M2:2
CHR17_M3:1
CHR17_M4:5
CHR17_M5:1
CHR17_M5:2
CHR17_M6:1
CHR17_M6:2
CHR17_M7:2
CHR17_M8:1
CHR17_M8:2
CHR17_M9:1
CHR17_M9:2

FIG. 6(B)

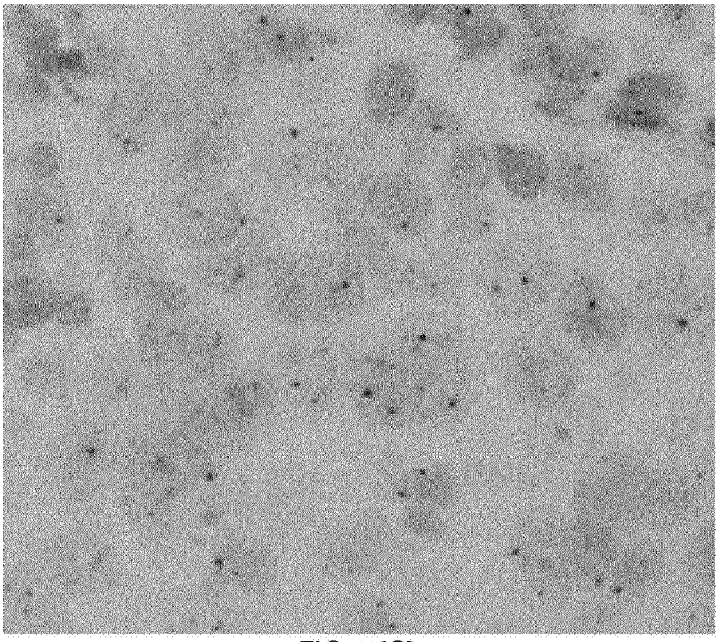

FIG. 6(C)

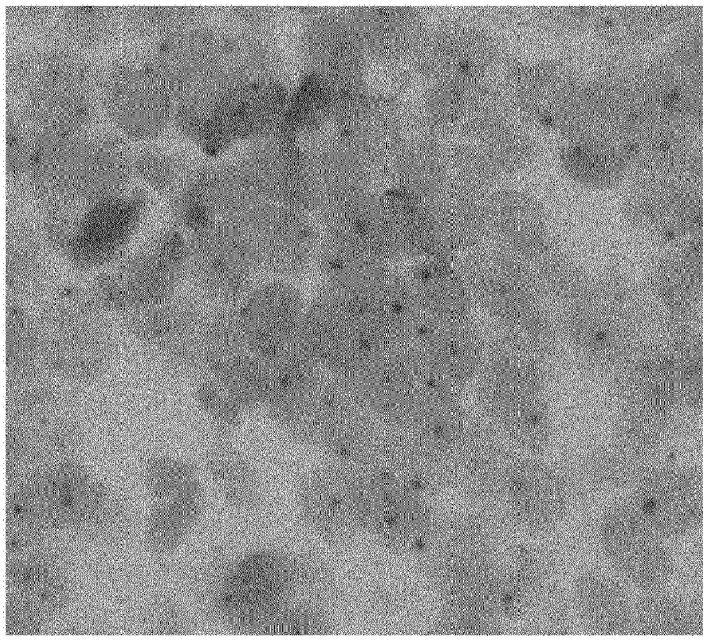

FIG. 6(D)

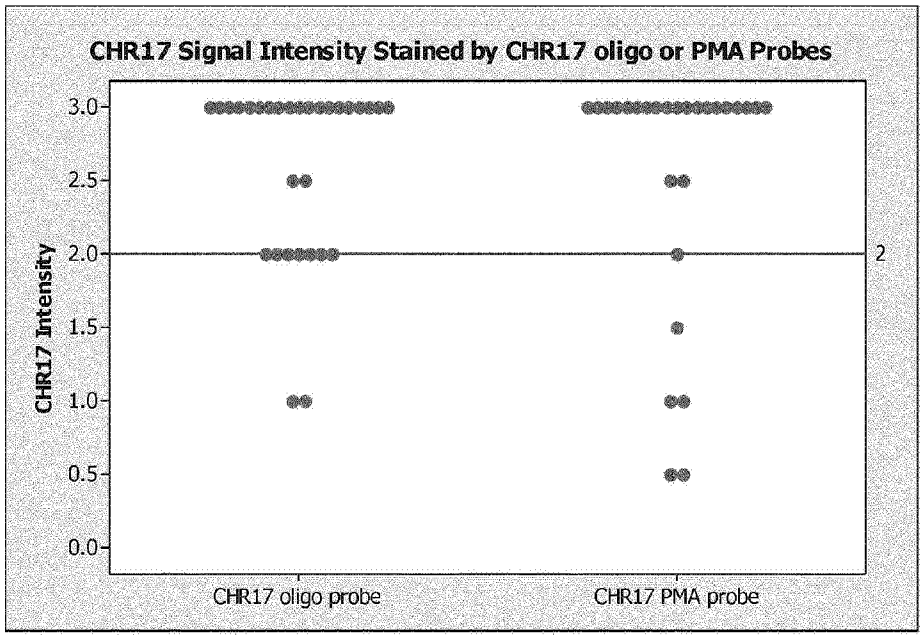
CHR17 Signal Intensity Stained by CHR17 oligo or PMA Probes
CHR17 Intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
CHR17 oligo probe
CHR17 PMA probe

FIG. 7(A)

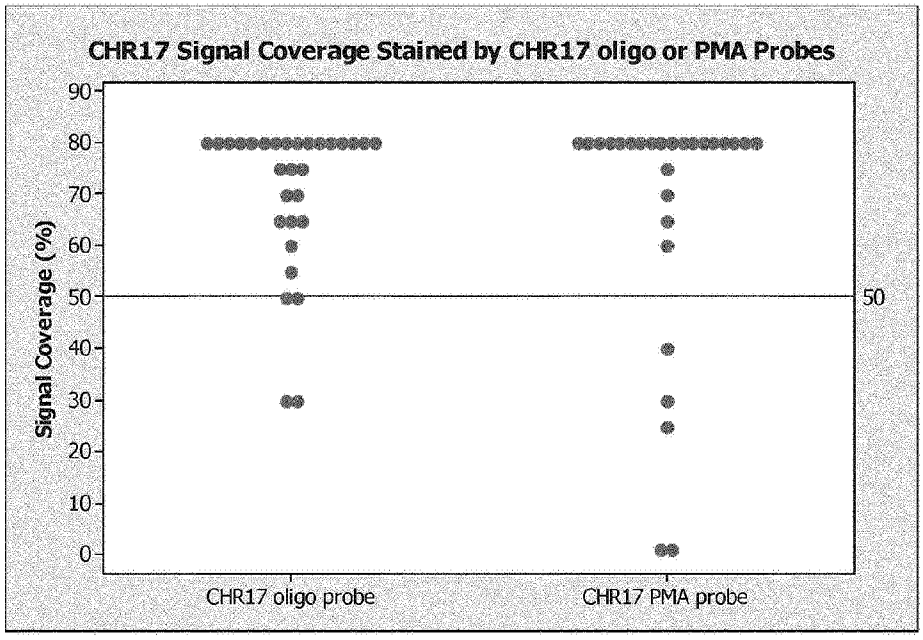
CHR17 Signal Coverage Stained by CHR17 oligo or PMA Probes
Signal Coverage (%)
90
80
70
60
50
40
30
20
10
0
50
CHR17 oligo probe
CHR17 PMA probe

FIG. 7(B)

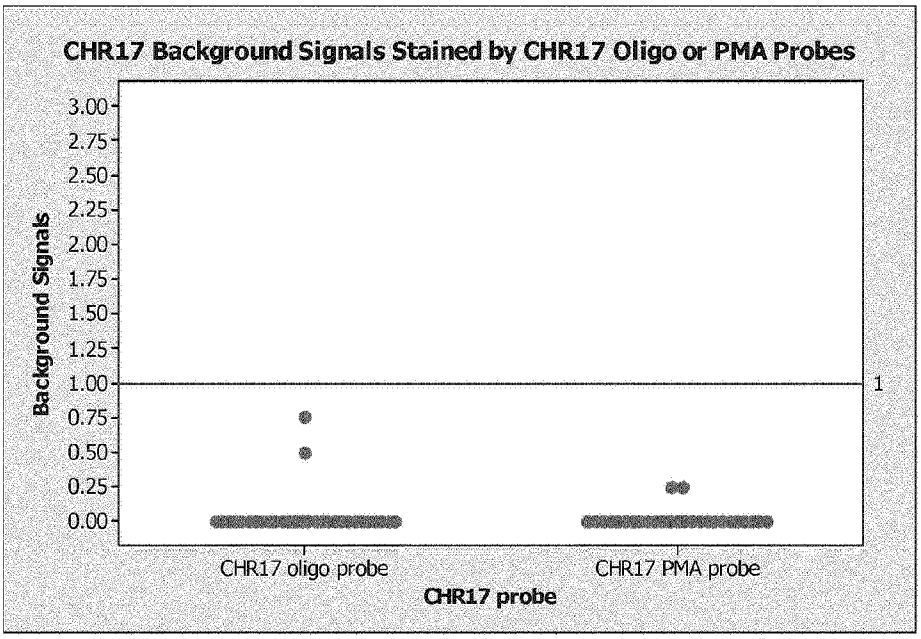
CHR17 Background Signals Stained by CHR17 Oligo or PMA Probes
Background Signals
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1
CHR17 oligo probe
CHR17 PMA probe
CHR17 probe

FIG. 7(C)

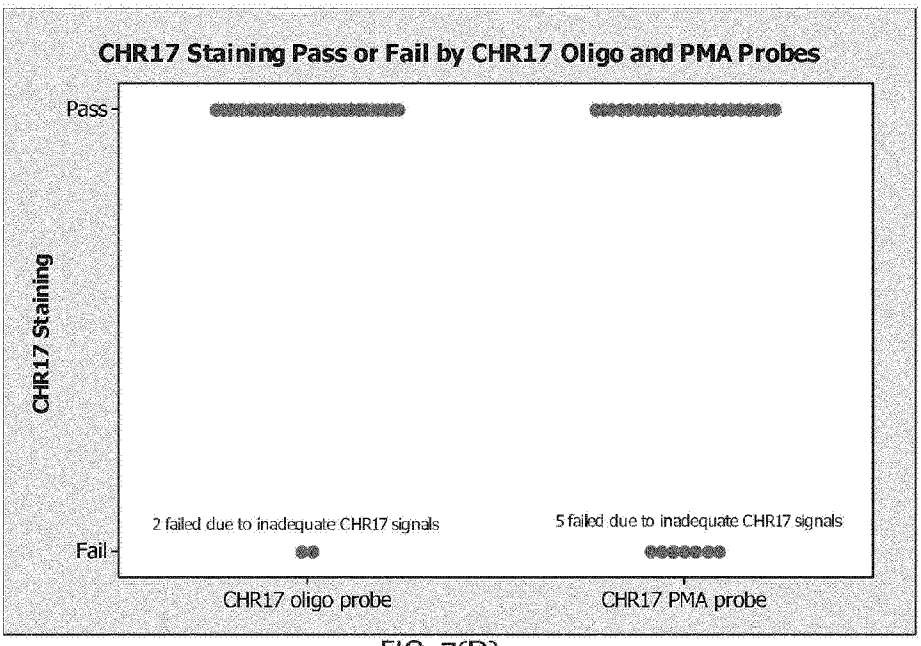
CHR17 Staining Pass or Fail by CHR17 Oligo and PMA Probes
CHR17 Staining
Pass
Fail
2 failed due to inadequate CHR17 signals
5 failed due to inadequate CHR17 signals
CHR17 oligo probe
CHR17 PMA probe

FIG. 7(D)

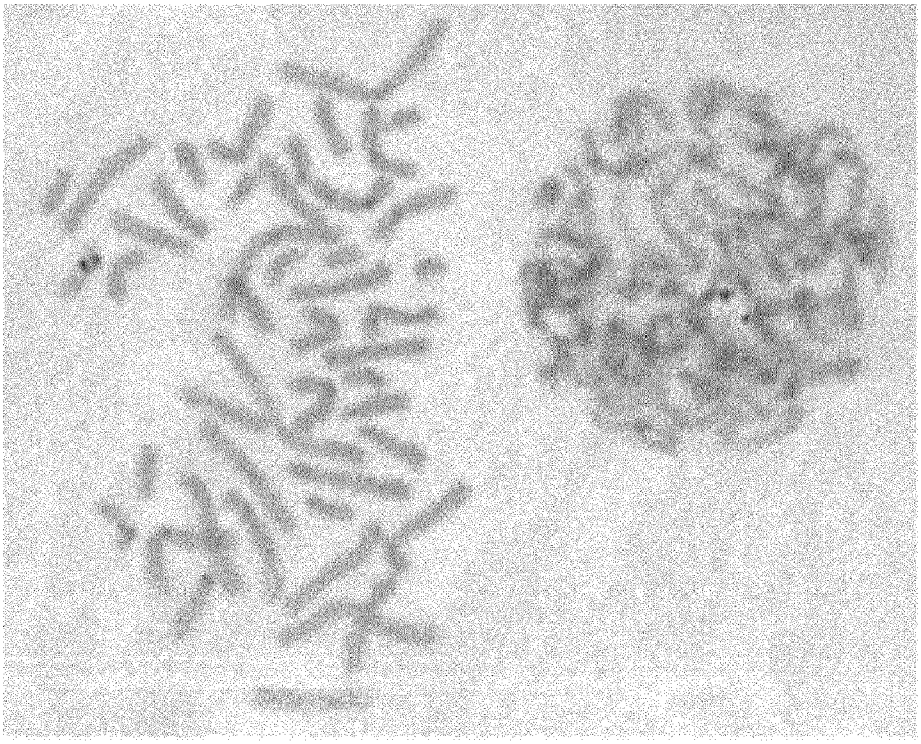

FIG. 8

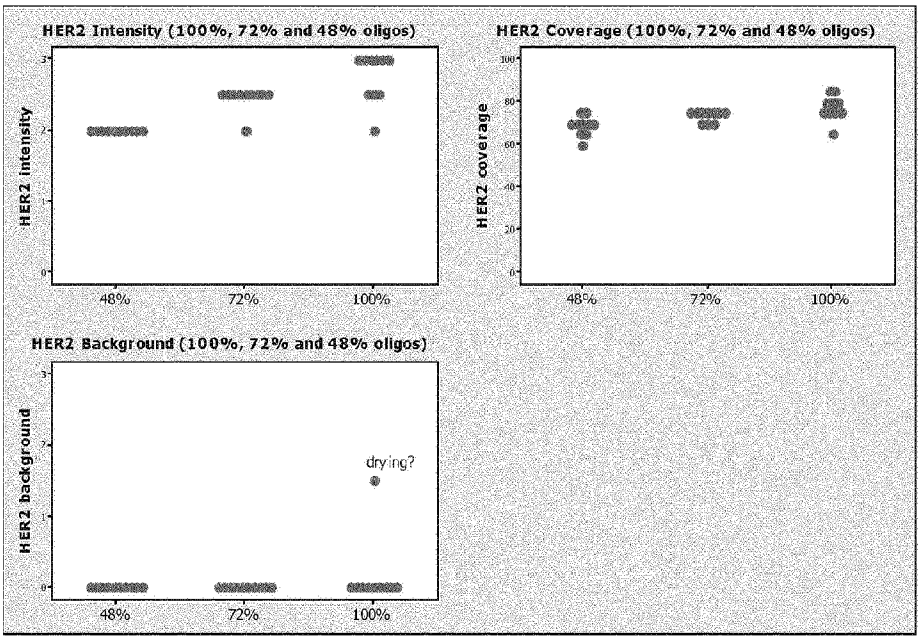
HER2 Intensity (100%, 72% and 48% oligos)
HER2 intensity
48%
72%
100%
HER2 Coverage (100%, 72% and 48% oligos)
HER2 coverage
48%
72%
100%
HER2 Background (100%, 72% and 48% oligos)
HER2 background
drying?
48%
72%
100%

FIG. 9

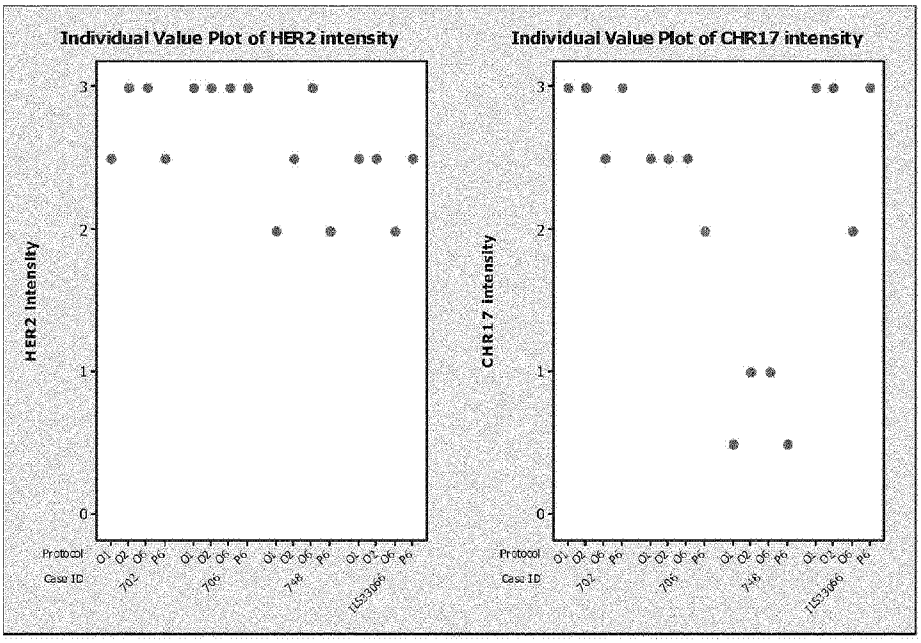
Individual Value Plot of HER2 intensity
HER2 Intensity
Protocol
Case ID
Individual Value Plot of CHR17 intensity
CHR17 intensity
Protocol
Case ID

FIG. 10

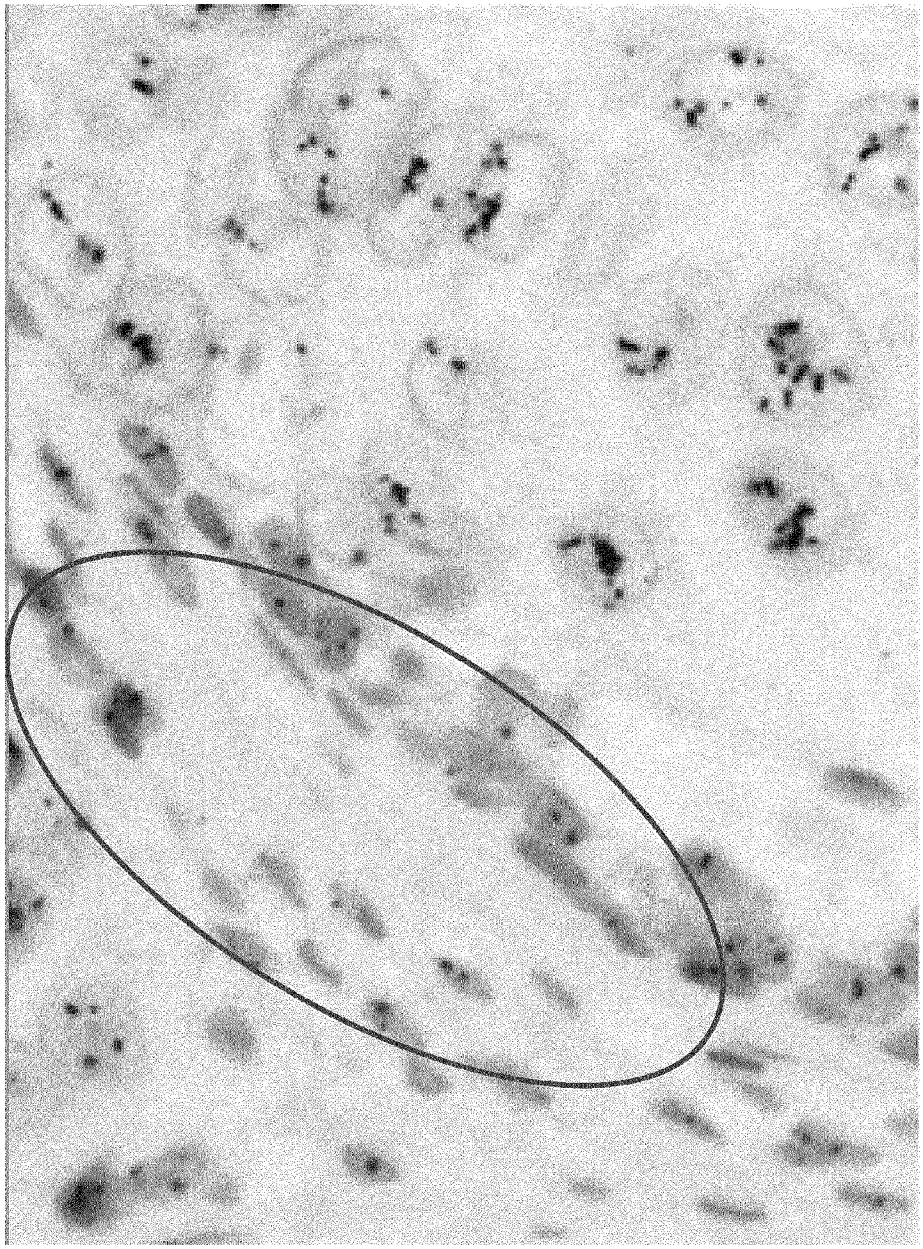

FIG. 11(A)

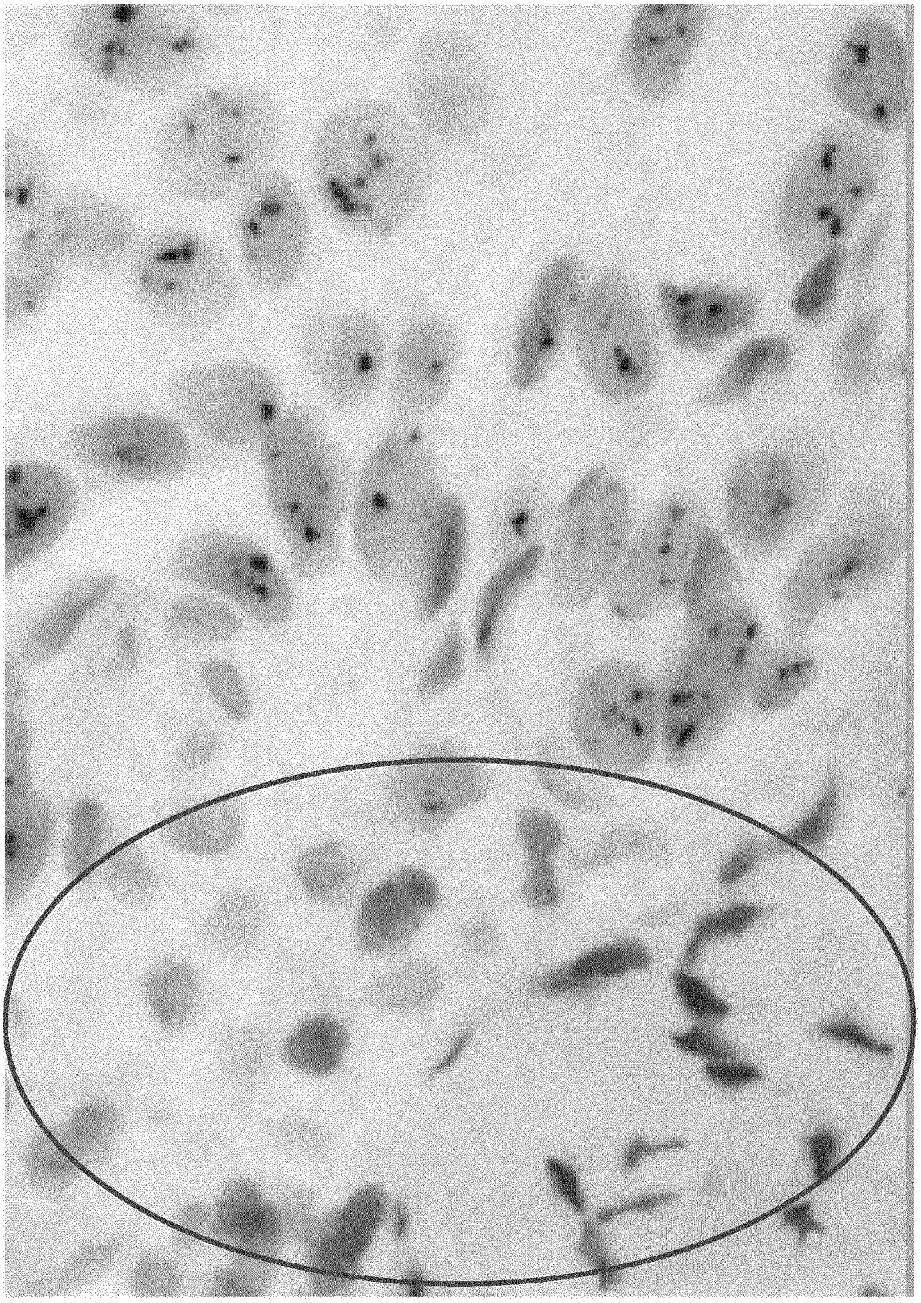

FIG. 11(B)

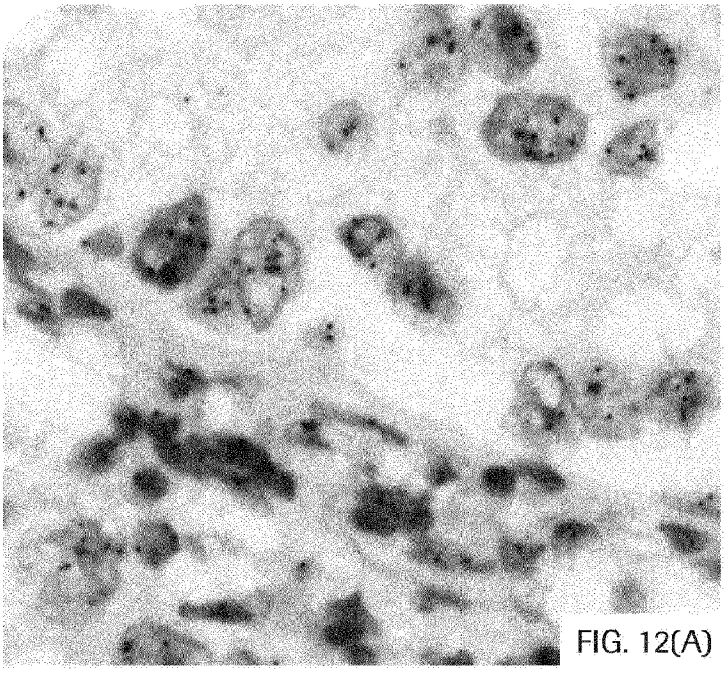

FIG. 12(A)

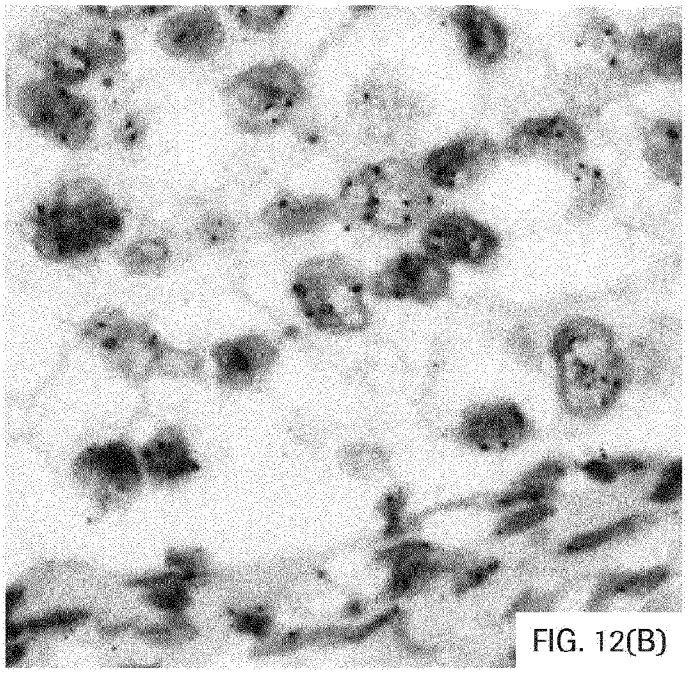

FIG. 12(B)

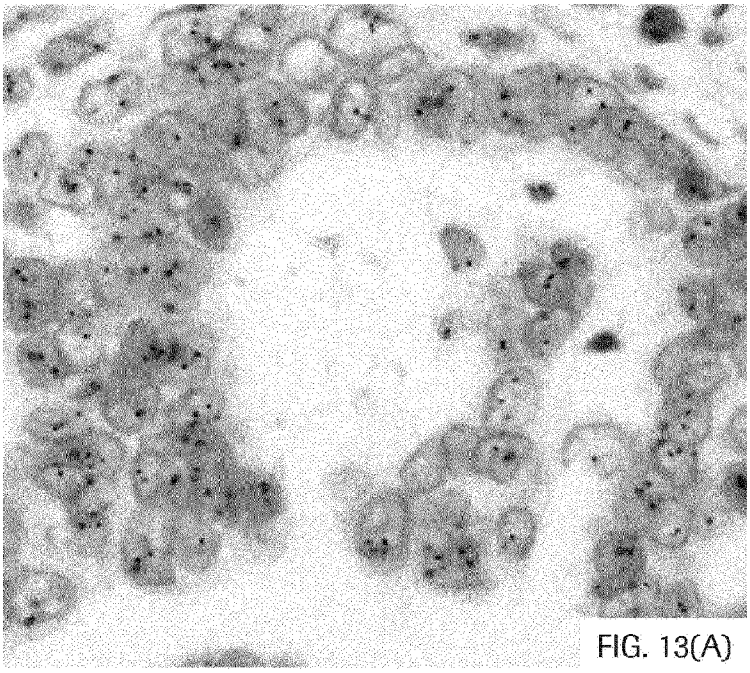

FIG. 13(A)

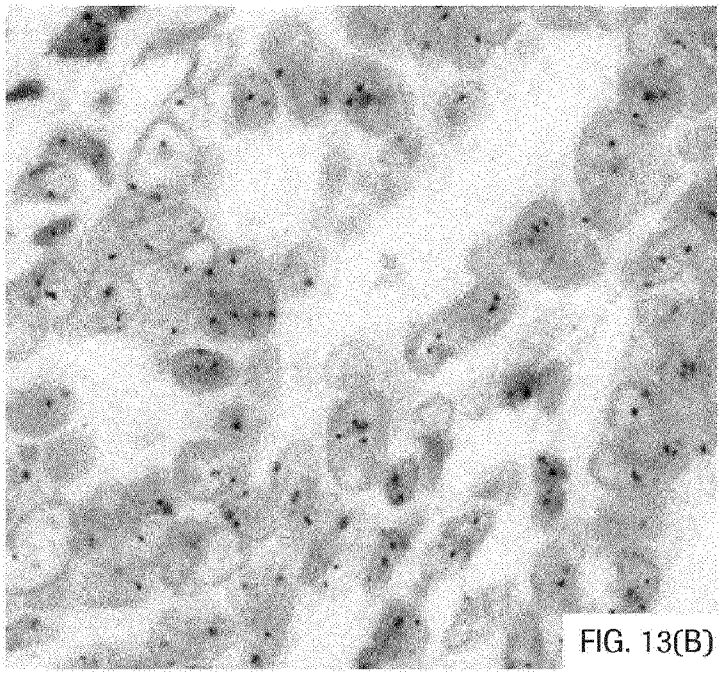

FIG. 13(B)

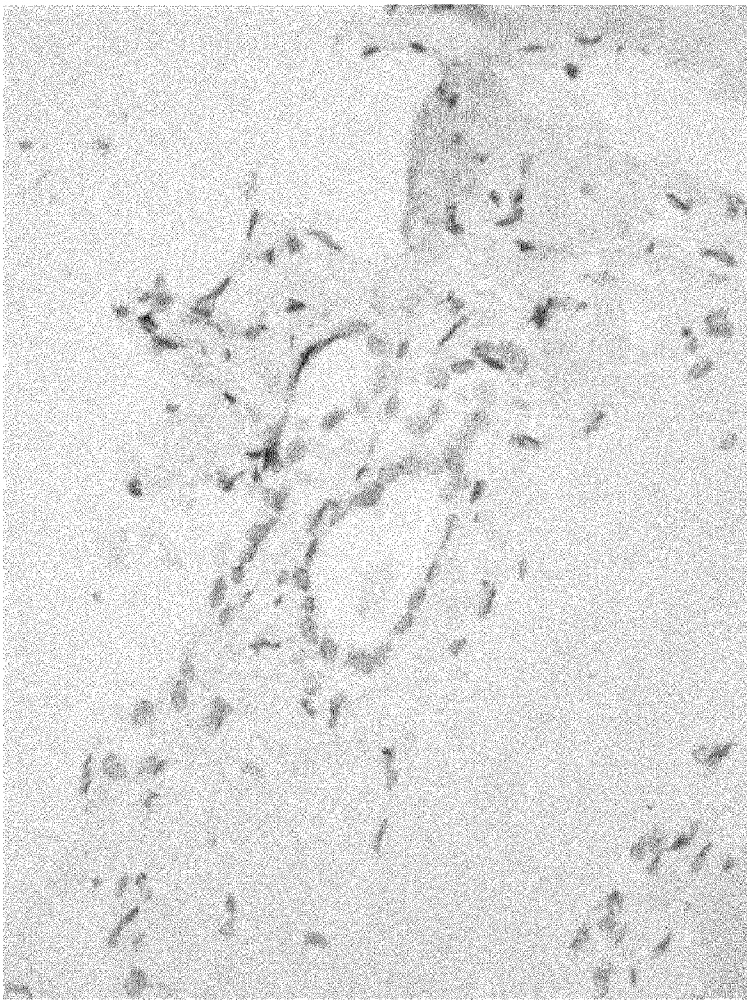

FIG. 14(A)

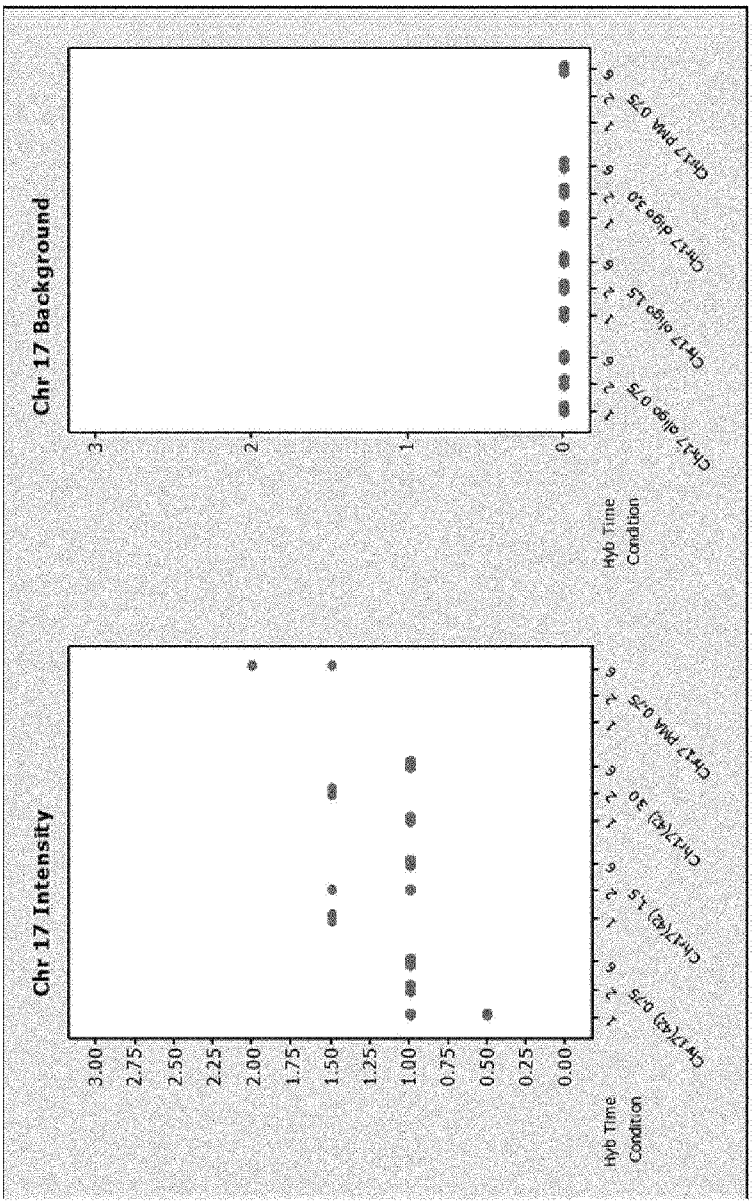
Chr 17 Intensity
Chr 17 Background
Hyb Time
Condition
Ch17 dUpp 0.75
Ch17 dUpp 1.5
Ch17 dUpp 3.0
Ch17 PMA 0.75
Ch17(dp) 0.75
Ch17(dp) 1.5
Ch17(dp) 3.0
Ch17 PMA 0.75

FIG. 14(B)

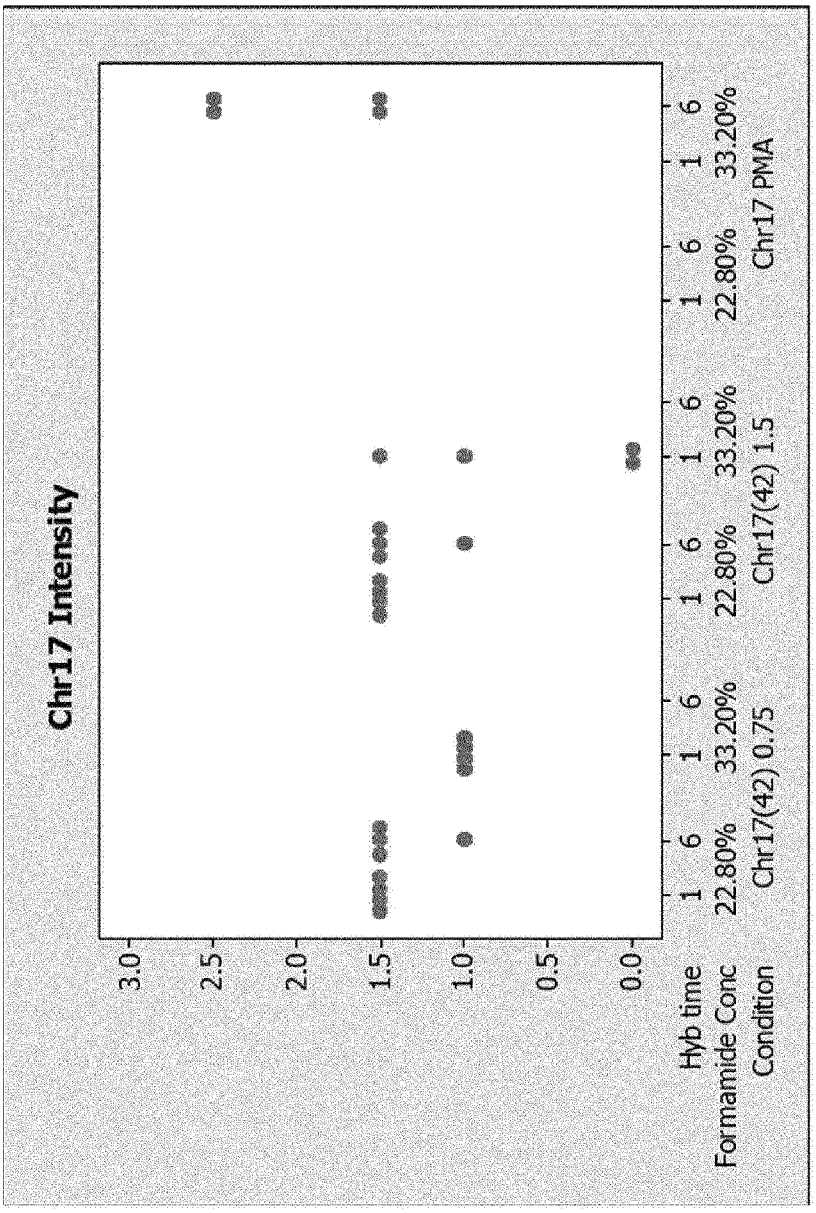
Chr17 Intensity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Hyb time 1 6 1 6 1 6 1 6 1 6 1 6
Formamide Conc 22.80% 33.20% 22.80% 33.20% 22.80% 33.20%
Condition Chr17(42) 0.75 Chr17(42) 1.5 Chr17 PMA

FIG. 14(C)

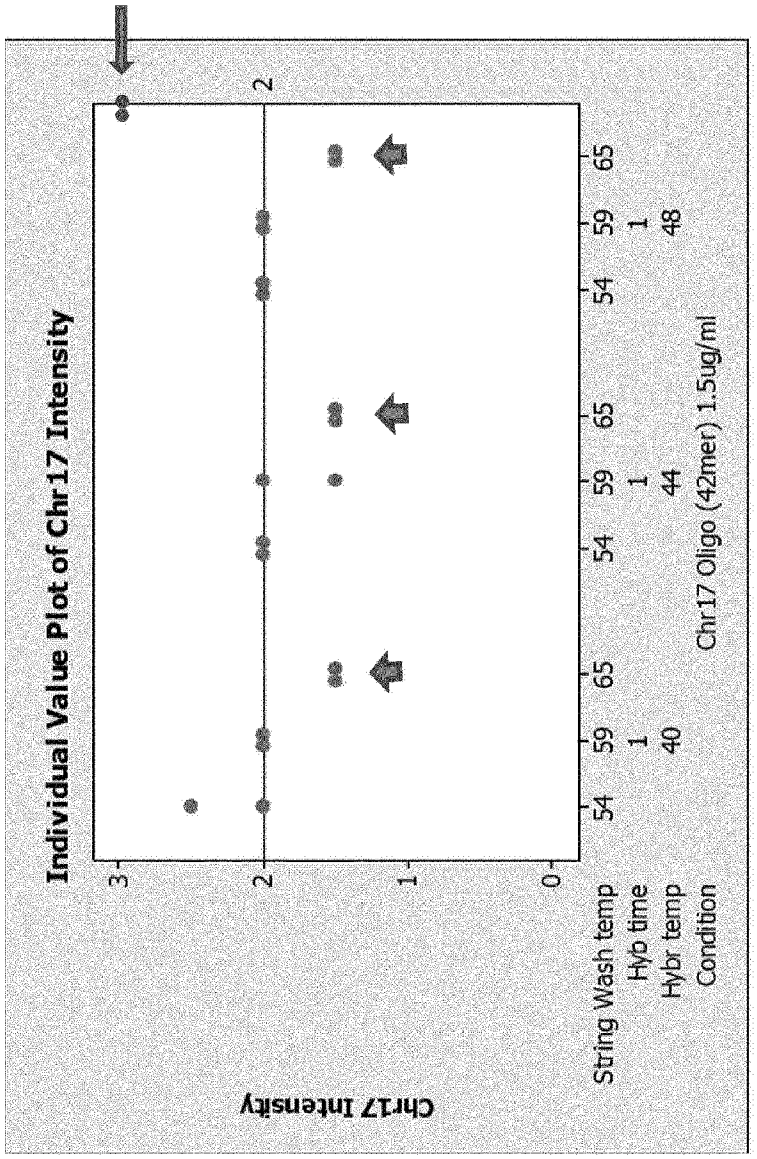
Individual Value Plot of Chr17 Intensity
Chr17 Intensity
3
2
1
0
String Wash temp
Hyb time
Hybr temp
Condition
54 59 65 54 59 65 54 59 65
1 1 1
40 44 48
Chr17 Oligo (42mer) 1.5ug/ml

FIG. 14(D)

CHR17 oligos	Length	# hits on CHR17	# off-target hits		
	nt	>85% identity	>85% identity	Continueous stretch	chromosomes
M1.1	79	23	5	>=78nt	1, 3, 20
M11.2	71	17	8	71nt	X, 1
M12.1	80	7	7	80nt	X
M13.1	80	19	18	>=78nt	1, 9, 20
M16.1	80	19	16	80 nt	1
M16.2	80	16	11	80nt	1
M2.1	79	21	33	79nt	1
M2.2	79	18	14	>=72nt	X
M3.1	79	23	16	>=70nt	22, 20, X, 2, 14, 11, 21, 3
M5.1	83	25	33	>=71nt	1
M5.2	87	16	22	>=76nt	1, 3, 16
M8.2	71	19	38	>=66nt	1, X, 14
M9.1	58	19	20	>=57nt	1, X, 11, 12
M9.2	65	17	7	>=58nt	1, X, 11, 22

FIG. 15

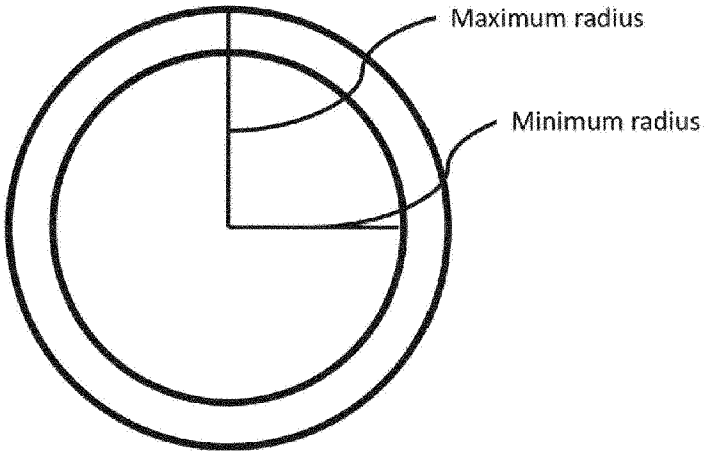

FIG. 16(A)

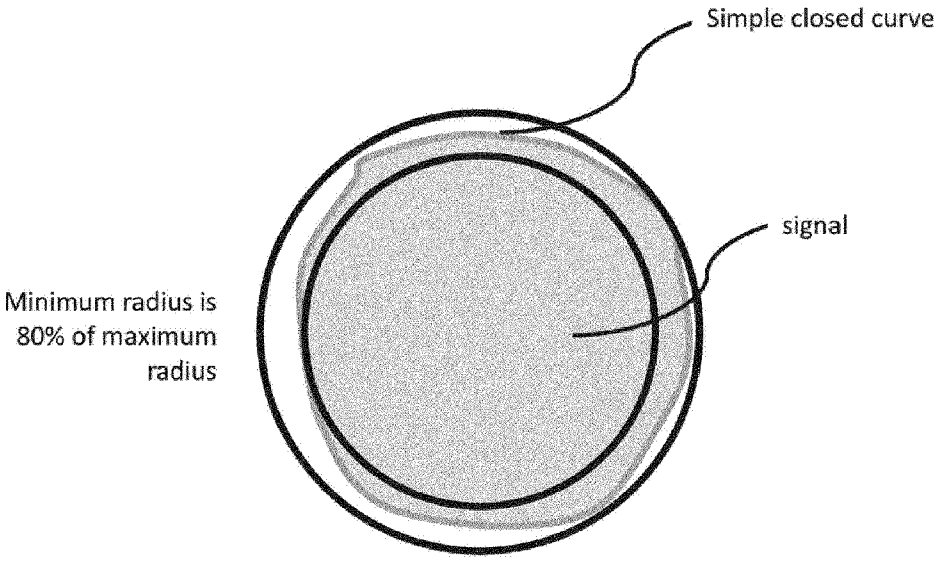

FIG. 16(B)

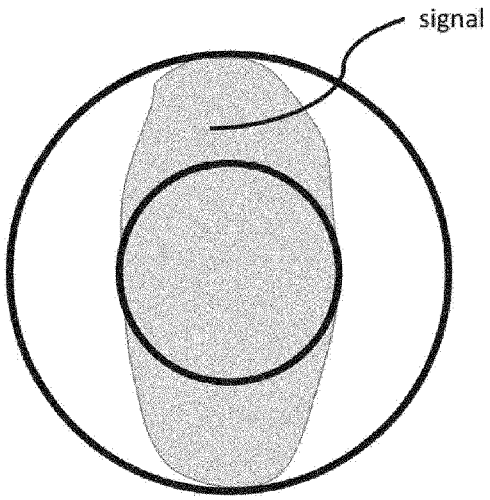

FIG. 16(C)

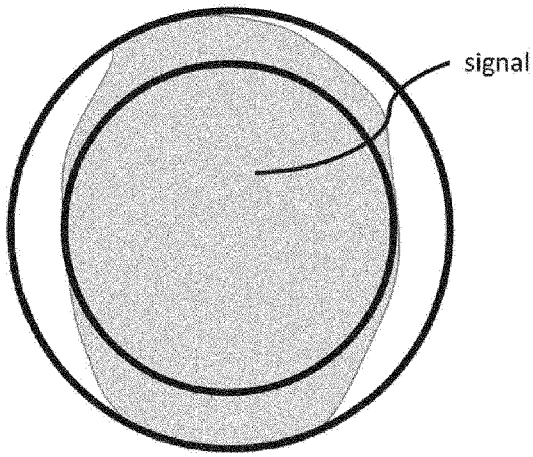

FIG. 16(D)