(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 July 2017 (13.07.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/120309 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2017/012298

(22) International Filing Date:
5 January 2017 (05.01.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/275,066 5 January 2016 (05.01.2016) US

(71) Applicant: **THE TEXAS A&M UNIVERSITY SYSTEM** [US/US]; 3369 TAMU, College Station, TX 77843-3369 (US).

(72) Inventors: **VAUGHN, Robert, N.**; 3369 TAMU, College Station, TX 77843-3369 (US). **STELLY, David, M.**; 3369 TAMU, College Station, TX 77843-3369 (US).

(74) Agent: **HANSON, Robert, E.**; Dentons US LLP, P.O. Box 061080, Wacker Drive Station, Willis Tower, Chicago, IL 60606-1080 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/120309 A1

(54) Title: COMPARATIVE LOCUS AMPLIFICATION FOR DETERMINING COPY NUMBER

(57) Abstract: The invention provides a rapid, accurate, sensitive, and low-cost method for determining the relative copy number of a nucleotide sequence from a biological sample. The method involves quantitation of a fluorescent marker in a test sample, and a different fluorescent marker in a control (reference) sample. Comparison of the amount of fluorescence between the two samples provides the relative copy number of the nucleotide sequence in the test sample, and can therefore be used to determine copy number variation.

TITLE

COMPARATIVE LOCUS AMPLIFICATION FOR DETERMINING COPY NUMBER

CROSS-REFERENCE TO RELATED INVENTIONS

This application claims the benefit of United States Provisional Application No. 62/275,066, filed January 5, 2016, herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to methods for determining the copy number of a nucleotide sequence of interest in a biological sample, more specifically to rapid and inexpensive methods for determining copy number or copy number variation of a nucleotide sequence of interest, for example a chromosome or gene, in a biological sample.

INCORPORATION OF SEQUENCE LISTING

The sequence listing that is contained in the file named "TAMC042WO_ST25.txt" which is 3.04 kilobytes as measured in Microsoft Windows operating system and was created on January 3, 2017, is filed electronically herewith and incorporated herein by reference.

BACKGROUND OF THE INVENTION

There is a current lack of rapid, accurate, sensitive and inexpensive methods for determining the copy number of a nucleotide sequence, such as a gene or other sequence. Current methods for determination of copy number, such as fluorescent *in situ* hybridization (FISH), comparative genomic hybridization (CGH), array comparative genomic hybridization, high-resolution melting (HRM), quantitative polymerase chain reaction (qPCR), digital PCR (dPCR) and next-generation sequencing (NGS) or high-throughput sequencing, each have one or more drawback, such as complexity, requirement for advanced or special equipment or skills, length of time required to perform the technique, and relatively high expense. Therefore, new methods are needed for determining the copy number of a particular nucleotide sequence.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method for determining copy number of a nucleotide sequence of interest in a nucleic acid sample, comprising the steps of: (a) amplifying

the nucleotide sequence of interest or a portion thereof in the nucleic acid sample using a first oligonucleotide primer comprising a 3' region that preferentially or specifically binds to said nucleotide sequence of interest or portion thereof and a 5' region that comprises a nucleotide sequence corresponding to a first fluorescent-labeled oligonucleotide primer and a second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the nucleic acid sample using a third oligonucleotide primer comprising a 3' region that preferentially or specifically binds to the control nucleotide sequence and a 5' region that comprises a nucleotide sequence corresponding to a second fluorescent-labeled oligonucleotide primer and a fourth oligonucleotide primer, in a reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer; and (b) comparing fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer with fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, to determine the copy number of the nucleotide sequence of interest. In one embodiment the nucleotide sequence of interest comprises genomic DNA, such as a portion of a chromosome, a gene or a regulatory element. In another embodiment the nucleic acid sample is from a plant, such as a cotton, sorghum, maize, tomato, rice, barley or wheat plant, or a mammal, for example a livestock animal such as a cow, pig, sheep, chicken, goat, horse, turkey, duck, or goose, a companion animal, such as a dog, cat, rabbit, guinea pig or hamster, or a human. In certain embodiments the first fluorescent-labeled oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer are labeled with two different fluorochromes or fluorescent dyes. Fluorochromes or fluorescent dyes for use in particular embodiments of the present invention are set forth in detail below. In other embodiments the first quenching oligonucleotide primer and the second quenching oligonucleotide primer is labeled with the same or different labels for quenching the fluorescence of the first fluorescent-labeled oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer. Labels for quenching the fluorescence of the fluorochromes or fluorescent dyes used for the first fluorescent-labeled oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer for use in exemplary embodiments of the present invention are set forth in detail below. In further embodiments, the method further comprises the use of a passive fluorescent marker as a fluorescence standard.

In an additional aspect, the present invention provides a method of determining copy number of a nucleotide sequence of interest in a first nucleic acid sample, comprising the steps of: (a) amplifying the nucleotide sequence of interest or a portion thereof in the first nucleic acid sample using a first oligonucleotide primer comprising a 3' region that specifically binds to said nucleotide sequence of interest or portion thereof and a 5' region that comprises a nucleotide sequence corresponding to a first fluorescent-labeled oligonucleotide primer and a second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the first nucleic acid sample using a third oligonucleotide primer comprising a 3' region that specifically binds to the control nucleotide sequence and a 5' region that comprises a nucleotide sequence corresponding to a second fluorescent-labeled oligonucleotide primer and a fourth oligonucleotide primer, in a first reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer; (b) amplifying the nucleotide sequence of interest or a portion thereof in a second nucleic acid sample using the first oligonucleotide primer and the second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the second nucleic acid sample using the third oligonucleotide primer and the fourth oligonucleotide primer, in a second reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer; (c) determining a first normalized fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer and a second normalized fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, utilizing fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer and fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer in the first and second reactions; and (d) comparing the first normalized fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer with the second normalized fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, to determine the copy number of the nucleotide sequence of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-10 sequence, based on normalization of the chromosome-10 sequence to a chromosome-16 sequence in *Gossypium hirsutum* L.

FIG. 2 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-10 sequence, based on normalization of the chromosome-10 sequence to a chromosome-20 sequence in *Gossypium hirsutum* L.

FIG. 3 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-11 sequence, based on normalization of the chromosome-11 sequence to a chromosome-20 sequence in *Gossypium hirsutum* L.

FIG. 4 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-20 sequence, based on normalization of the chromosome-20 sequence to a chromosome-10 sequence in *Gossypium hirsutum* L.

FIG. 5 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-20 sequence, based on normalization of the chromosome-20 sequence to a chromosome-11 sequence in *Gossypium hirsutum* L.

FIG. 6 – Shows the results of comparative locus amplification (CLA) for a "gBlock" dilution series for a chromosome-11 sequence from *Gossypium hirsutum* L, using UBC1 as the reference sequence.

FIG. 7 –Shows the results of comparative locus amplification (CLA) analysis on euploid TM1 genetic/cytogenetic standard and chromosome-11 monosomic aneuploids ("H11") to determine where they fell relative to the synthetic "gBlock" dilutions.

FIG. 8 – Shows the results of a dilution series of comparative locus amplification (CLA) assays to test the sensitivity of FAM/HEX signal ratios to starting concentration of sample template DNA.

FIG. 9 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome 3 sequence, using UBC1 as the reference sequence.

FIG. 10 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome 7 sequence, using UBC1 as the reference sequence.

FIG. 11 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-10 sequence, using UBC1 as the reference sequence.

FIG. 12 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-11 sequence, using UBC1 as the reference sequence.

FIG. 13 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-16 sequence, using SAD1 as the reference sequence.

FIG. 14 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-21 sequence, using UBC1 as the reference sequence.

FIG. 15 – Shows the results of comparative locus amplification (CLA) to determine the copy number of a chromosome-25 sequence, using UBC1 as the reference sequence.

FIG. 16 – Shows the results of comparative locus amplification (CLA) for a transgene insert in a first transformed line, using UBC1 as the reference sequence.

FIG. 17 – Shows the results of comparative locus amplification (CLA) for a transgene insert in a second transformed line, using UBC1 as the reference sequence.

FIG. 18 – Shows the results of comparative locus amplification (CLA) for a transgene insert in a first transformed line, using UBC1 as the reference sequence.

FIG. 19 – Shows the results of comparative locus amplification (CLA) for a transgene insert in a second transformed line, using UBC1 as the reference sequence.

FIG. 20 – Shows the results of comparative locus amplification (CLA) for a *Gossypium* insert in a first transformed line, using UBC1 as the reference sequence.

FIG. 21 – Shows the results of comparative locus amplification (CLA) for a *Gossypium* insert in the Coker 312 parent line, using UBC1 as the reference sequence.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NOs:1-16 – Primers used for CLA to determine the copy number of chromosomes in *Gossypium hirsutum* L.

DETAILED DESCRIPTION

The present invention provides methods for determining the relative copy number, or copy number variation (CNV), in a biological sample using comparative locus amplification (CLA).

Methods currently available in the art for determination of the copy number or copy number variation of a nucleotide sequence in biological samples are relatively expensive, time-consuming, require advanced or special equipment or skills, and can be relatively complex. The present invention improves upon one or more of the shortcomings of each of these methods and thus represents a significant improvement over the art, especially as related to simplicity, costs for required equipment and expendables. Comparative Locus Amplification (CLA) presents alternative means of detecting or determining copy number variation (CNV). Given that CNV detection/determination is applied in various situations (*e.g.*, survey, research, monitoring, diagnostic) that differ widely in relative requirement for accuracy, speed, personnel qualifications, equipment, expendables, *etc.*, the CLA methods provided herein fill certain niches better that all existing methods of CNV analysis. As described herein, the present invention uses a fluorochrome-based reporting system, such as fluorescence resonance energy transfer (FRET), to quantitate the relative amounts of two non-allelic target nucleotide sequences, and thus can be used to determine copy number, as well as copy number differences and CNV of nucleotide sequences in a biological sample. The methods utilize locus-specific and sequence-distinct primer pairs tagged with generic oligonucleotides that upon polymerase chain reaction (PCR) amplification lead to the release from quenching of two (or more) diagnostic fluorochromes and leads to fluorescence at two wavelengths that are largely locus-specific. The relative amplitudes of the two fluorescent markers will vary according to the relative copy number of the "target" and "control" (reference) nucleotide sequences. Based on the fluorescence amplitude ratio, it can be determined if the target nucleotide sequence is present in a higher or lower copy number than the control nucleotide sequence, making it a convenient and cost-effective method for rapid and accurate determination of the copy number or copy number variation of a nucleotide sequence in

a biological sample. Additionally, the methods described herein require no special or advanced expertise or equipment, in contrast to certain currently available methods.

CNVs are heritable differences that can be associated with genetic, genomic and phenotypic variation (such as flowering time, plant height and resistance to various biotic/abiotic stresses in plants), and thus are important in many areas of biology, for example to plant and animal breeders, as well as in medical diagnostics and treatment. Rapid and accurate determination of the copy number of a nucleotide or nucleic acid sequence in organisms, tissues or cells can be problematic. This is especially true in polyploid plants due to increased copy number of identical or similar DNA sequences. The presently disclosed CLA techniques facilitate germplasm characterization, genome characterization and germplasm manipulation, *e.g.*, germplasm surveys for CNV, genome sequence assembly and identification of various types of aneuploids. Additionally, the CLA system allows for CNV analysis for breeders of a variety of commercially significant crops and animals to select the parents for breeding in a targeted method that is rapid and relatively inexpensive. In diagnostics the presently described CLA system can be used to screen for CNV (for example, aneuploidy, segmental duplications and deletions), *e.g.*, in relationship to specific diseases or cancers. Being PCR-based, it can be applied to minute samples and is amenable to portable delivery.

CLA has a large number of context-specific applications, such as facilitating the selection of variants during breeding of commercially significant crops or animals, thus facilitating the development of new commercial lines with valuable traits. Additionally CLA can be used to discover or identify novel genetic variants and to introduce them into elite breeding germplasm, *e.g.*, for agriculturally important traits. CLA allows for the development of new lines in a way that otherwise would be very expensive and/or difficult. CLA allows breeders to screen a large number of progeny (*e.g.*, seeds) for a particular CNV of interest and select out only those carrying the CNV of interest, reducing the costs and generation time for advancing various traits related to these CNVs.

The methods described herein may be used to test a multitude of biological samples, for example tissues or cells from a plant or animal. As used herein, a "biological sample" or "sample" may also include clinical samples such as blood and blood parts including, but not limited to, serum, plasma, platelets, or red blood cells; sputum, mucosa, tissue, cultured cells, including primary cultures, explants, and transformed cells; and biological fluids. A biological

sample may also include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. A biological sample may be obtained from a eukaryotic organism, for example a plant, including cotton, sorghum, maize, tomato and wheat, or a mammal, including humans, cows, pigs, chickens, turkeys, ducks, geese, dogs, goats, and the like. Any tissue appropriate for use in accordance with the invention may be used, for instance, plant leaves, seeds, roots or stems, or animal skin, brain, spinal cord, adrenals, pectoral muscle, lung, heart, liver, crop, duodenum, small intestine, large intestine, kidney, spleen, pancreas, adrenal gland, bone marrow, lumbosacral spinal cord, or blood.

Nucleic acids, such as DNA or RNA, for use in the described methods may be isolated using any method available as would be known by one of skill in the art. In certain embodiments, a commercially available kit, such as the NucleoSpin® Plant II kit (MACHERY-NAGEL) or the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Life Technologies) may be used to isolate DNA from a biological sample. The isolated DNA can be assessed for quality and quantity using any of the numerous methods known to those of skill in the art. For example, the absorbance of the DNA sample at 230 nm, 260 nm and 280 nm can be obtained using a DS-11 photospectrometer (DeNovix®, Inc.), and determining the ratio of the absorbance at 260 nm to the absorbance at 280 nm, and the ratio of the absorbance at 260 nm to the absorbance at 230 nm (DNA samples having ratios of approximately 1.8 and 2.0, respectively, can be used in certain embodiments of the present invention).

Amplification of DNA

Methods such as polymerase chain reaction (PCR and RT-PCR) may be used to amplify nucleic acid sequences directly from genomic material, such as genomic DNA, mRNA, cDNA, or from genomic libraries, or cDNA libraries. It will be appreciated that, although many specific examples of primers are provided herein, suitable primers to be used with the invention can be designed using any suitable method. It is not intended that the invention be limited to any particular primer or primer pair.

In some embodiments, the fluorescent-labeled primers incorporate two different fluorescent labels or markers, while in other embodiments a passive fluorescent dye or fluorochrome is used. Suitable fluorescent labels, markers, dyes or fluorochromes for use in certain embodiments of the present invention include, but are not limited to, FAM™, 5-FAM,

6-FAM™, TET™, JOE™, VIC®, HEX™, NED™, PET®, ROX™, TAMRA™, TET™, Texas Red®, CAL Fluor® Gold 540, CAL Fluor® Orange 560, CAL Fluor® Red 590, CAL Fluor® Red 610, CAL Fluor® Red 635, Cy® (cyanine) 3, Cy® 3.5, Cy® 5, Cy® 5.5, Cy® 7, Cy® 7.5, Quasar® 570, Quasar® 670, Quasar® 705, Oyster®-500, Oyster®-550 P, Oyster®-550 D, Oyster®-556, Oyster®-645, Oyster®-650 P, Oyster®-650 D, Oyster®-656, phycoerythrin, allophycocyanin, TMR, TMRIC, Rhodamine, Rhodamine Green™, Rhodamine Red™, LightCycler® Red 610, LightCycler® Red 640, LightCycler® Red 670, Oregon Green® 488, Oregon Green® 500, Oregon Green® 514, Alexa Fluor® 350, Alexa Fluor® 405, Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 647, Alexa Fluor® 680, Alexa Fluor® 750, BODIPY® FL, BODIPY® 530/550, BODIPY® TMR-X, BODIPY® 630/650, BODIPY® 650/656, Fluorescein, Fluorescein isothiocyanate (FITC), Fluorescein-dT, Pacific Blue™, Pacific Green™, Pacific Orange™, Yakima Yellow™, DY-405, DY-415, DY-430, DY-431, DY-478, DY-480XL, DY-490, DY-495, DY-505, DY-530, DY-547, DY-547P1, DY-548, DY-549, DY-549P1, DY-550, DY-554, DY-555, DY-556, DY-560, DY-590, DY-591, DY-594, DY-605, DY-610, DY-615, DY-630, DY-631, DY-632, DY-633, DY-634, DY-635, DY-636, DY-647, DY-647P1, DY-648, DY-648P1, DY-649, DY-649P1, DY-650, DY-651, DY-652, DY-654, DY-675, DY-676, DY-677, DY-678, DY-679P1, DY-680, DY-681, DY-682, DY-700, DY-701, DY-703, DY-704, DY-730, DY-731, DY-732, DY-734, DY-749, DY-749P1, DY-750, DY-751, DY-752, DY-754, DY-776, DY-777, DY-778, DY-780, DY-781, DY-782, DY-800, DY-831, ATTO 390, ATTO 425, ATTO 465, ATTO 488, ATTO 495, ATTO 514, ATTO 520, ATTO 532, ATTO Rho6G, ATTO 542, ATTO 550, ATTO 565, ATTO Rho3B, ATTO Rho11, ATTO Rho12, ATTO Thio12, ATTO Rho101, ATTO 590, ATTO 594, ATTO Rho13, ATTO 610, ATTO 620, ATTO Rho14, ATTO 633, ATTO 647, ATTO 647N, ATTO 655, ATTO Oxa12, ATTO 665, ATTO 680, ATTO 700, ATTO 725, and ATTO 740.

In certain embodiments, the quenching oligonucleotide primers incorporate a quencher label for the fluorescent labels or markers. Suitable quencher labels for use in certain embodiments of the present invention include, but are not limited to, Deep Dark Quencher I (DDQI), Deep Dark Quencher II (DDQII), dabcyl, Eclipse™, Iowa Black® FQ, Iowa Black® RQ, Black Hole Quencher-0 (BHQ-0), Black Hole Quencher-1 (BHQ-1), Black Hole

Quencher-2 (BHQ-2), Black Hole Quencher-3 (BHQ-3), Black Hole Quencher-10 (BHQ-10), QSY®-7 or QSY®-21.

It is not intended that the primers of the invention be limited to generating an amplicon of any particular size. For example, the primers used to amplify a region of a chromosome, a chromosome fragment, gene or sequence described herein are not limited to amplifying the entire region of a relevant locus. A primer can generate an amplicon of any suitable length that is longer or shorter than those disclosed herein. In some embodiments, amplification of a target sequence may produce an amplicon at least 20 nucleotides in length, or alternatively, at least 50 nucleotides in length, or alternatively, at least 100 nucleotides in length, or alternatively, at least 200 nucleotides in length. Target sequences in addition to those recited herein may also find use with the present invention.

Primers for use in the present invention may be any length sufficient to hybridize to and enable amplification of a nucleic acid as described herein, including at least or about 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, 30 nucleotides, 35 nucleotides, 40 nucleotides, 45 nucleotides, or 50 nucleotides; or from about 12 to about 50 nucleotides in length, 15 to 30 nucleotides in length, 15 to 25 nucleotides in length, or 20 to 30 nucleotides in length. DNA primers suitable for use with the present invention may be any primers described herein, such as those set forth as SEQ ID NOs:1-16.

A PCR assay may include a number of reagents and components, including a master mix comprising certain primers that are labeled with a fluorescent nucleic acid dye or quencher, as detailed herein. In some embodiments, an exemplary PCR master mix may contain template nucleic acid material, such as DNA, PCR primers, salts such as $MgCl_2$, a polymerase enzyme, such as Taq polymerase, deoxyribonucleotides, and one or more buffer. One of skill in the art will be able to identify useful components of a master mix in accordance with the present invention. In one embodiment, a master mix such as KASP™ Master Mix (LCG Ltd.), which contains the high resolution melting (HRM) dye, SYTO®9 may be used.

During real-time PCR detection, PCR may be performed in any reaction volume, such as 10 μL, 20 μL, 30 μL, 50 μL, 100 μL, or the like. Reactions may be performed singly, in duplicate, or in triplicate. PCR thermal cycling conditions are well known in the art and vary based on a number of factors. As described herein, an exemplary amplification protocol may include, for example, an initial denaturation at 94°C for 15 minutes; 10 cycles of 94°C for 20 seconds, 55-61°C for 60 seconds (-0.6°C per cycle); and 18 cycles +2 until the optimum is reached at 94°C for 20 seconds, 55°C for 60 seconds. Any thermal cycling program may be designed as appropriate for use with the particular primers for detection of particular nucleic acid species as would be understood by one of skill in the art.

Test samples or assays as described herein may be compared to a control or reference sample, such as a copy number control, in order to accurately determine the copy number of the test sample. In addition, a reaction control may be used to avoid false negative results and thereby increase the reliability of an assay. Use of a reaction control provides assurance that a negative result for a target is truly a negative result rather than due to a problem or break-down in the reaction. Because the signal for the reaction control should always be generated, even when the target signal is not generated (*i.e.*, the target DNA is not present in the sample), this would indicate that a negative target signal is indeed a negative result. A reaction control may be useful in diagnostic assays because certain biological samples may harbor inhibitory components that may interfere with PCR amplification, leading to false negative results. In addition, control reactions without any DNA can be utilized to rule out false positive results that could result from contamination of one or more of the reaction components, and/or interactions of reaction components, *e.g.*, primer sets.

As used herein, the term "complementary nucleic acids" refers to two nucleic acid molecules that are capable of specifically hybridizing to one another, wherein the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. In this regard, a nucleic acid molecule is said to be the complement of another nucleic acid molecule if they exhibit complete complementarity. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be complementary if they can hybridize to one another with sufficient stability to

permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are known in the art.

Departures from complete complementarity are permissible, as long as the capacity of the molecules to form a double-stranded structure remains. Thus, in order for a nucleic acid molecule or a fragment of the nucleic acid molecule to serve as a primer or probe, such a molecule or fragment need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed. A nucleotide sequence when observed in the 5' to 3' direction is said to be a "complement" of, or complementary to, a second nucleotide sequence observed in the 3' to 5' direction if the first nucleotide sequence exhibits complete complementarity with the second or reference sequence. As used herein, nucleic acid sequence molecules are said to exhibit "complete complementarity" when every nucleotide of one of the sequences read 5' to 3' is complementary to every nucleotide of the other sequence when read 3' to 5'. A nucleotide sequence that is complementary to a reference nucleotide sequence will exhibit a sequence identical to the reverse complement sequence of the reference nucleotide sequence.

Diagnostic Tests and Kits

The invention further provides diagnostic reagents and kits comprising one or more such reagents or components for use in a variety of diagnostic assays, including for example, nucleic acid assays, *e.g.*, PCR or RT-PCR assays. Such kits may preferably include at least a first and second primer pair as described herein, and means for detecting amplification of a target and control sequence. In some embodiments, such a kit may contain multiple primer pairs as described herein for the purpose of detection of multiple target sequences. Primer pairs may be provided in lyophilized, dessicated, or dried form, or may be provided in an aqueous solution or other liquid media appropriate for use in accordance with the invention.

Kits may also include additional reagents, *e.g.*, PCR components, such as salts including $MgCl_2$, a polymerase enzyme, and deoxyribonucleotides, and the like, reagents for DNA isolation, or enrichment of a biological sample, including for example media such as water, saline, or the like, as described herein. Such reagents or components are well known in the art. Where appropriate, reagents included with such a kit may be provided either in the same container or media as the primer pair or multiple primer pairs, or may alternatively be placed in a second or additional distinct container into which the additional composition or reagents may be

placed and suitably aliquoted. Alternatively, reagents may be provided in a single container means.

Definitions

As used herein, the term "nucleic acid" refers to a single or double-stranded polymer of deoxyribonucleotide bases or ribonucleotide bases read from the 5' to the 3' end, which may include genomic DNA, target sequences, primer sequences, or the like. In accordance with the invention, a "nucleic acid" may refer to any DNA or nucleic acid to be used in an assay as described herein, which may be isolated or extracted from a biological sample. The term "nucleotide sequence" or "nucleic acid sequence" refers to both the sense and antisense strands of a nucleic acid as either individual single strands or in the duplex. The terms "nucleic acid segment," "nucleotide sequence segment," or more generally, "segment," will be understood by those in the art as a functional term that includes genomic sequences, target sequences, operon sequences, and smaller engineered nucleotide sequences that express or may be adapted to express, proteins, polypeptides or peptides. The nomenclature used herein is that required by Title 37 of the United States Code of Federal Regulations §1.822 and set forth in the tables in WIPO Standard ST.25 (1998), Appendix 2, Tables 1 and 3.

The term "gene" refers to components that comprise bacterial DNA or RNA, cDNA, artificial bacterial DNA polynucleotide, or other DNA that encodes a bacterial peptide, bacterial polypeptide, bacterial protein, or bacterial RNA transcript molecule, introns and/or exons where appropriate, and the genetic elements that may flank the coding sequence that are involved in the regulation of expression, such as, promoter regions, 5' leader regions, 3' untranslated region that may exist as native genes or transgenes in a bacterial genome. The gene or a fragment thereof can be subjected to polynucleotide sequencing methods that determines the order of the nucleotides that comprise the gene. Polynucleotides as described herein may be complementary to all or a portion of a bacterial gene sequence, including a promoter, coding sequence, 5' untranslated region, and 3' untranslated region. Nucleotides may be referred to by their commonly accepted single-letter codes.

The term "about" is used herein to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value. The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to

only alternatives and to "and/or." When not used in conjunction closed wording in the claims or specifically noted otherwise, the words "a" and "an" denote "one or more."

The terms "comprise," "have," and "include" are open-ended linking verbs. Any forms or tenses of one or more of these verbs, such as "comprises," "comprising," "has," "having," "includes," and "including," are also open-ended. For example, any method that "comprises," "has" or "includes" one or more steps is not limited to possessing only those one or more steps and also covers other unlisted steps. Similarly, any cell that "comprises," "has" or "includes" one or more traits is not limited to possessing only those one or more traits and covers other unlisted traits.

While the invention has been described in connection with specific embodiments thereof, it will be understood that the present invention is capable of further modifications by one of skill in the art. It is to be understood that, unless otherwise indicated, the present disclosure is not limited to particular materials, reagents, reaction materials, manufacturing processes, or the like, as such can vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It is also possible in the present disclosure that steps can be executed in different sequence where this is logically possible. The present disclosure is therefore intended to encompass any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth.

EXAMPLES

Example 1

CLA Analysis to Determine Copy Number of Chromosome-10 Sequence Based on Normalization to a Chromosome-16 Sequence in Cotton

In the present example CLA analysis results are shown for two kinds of cotton (*Gossypium hirsutum* L.), one with a normal complement (2n=52) comprising 26 pairs of homologous chromosomes (euploid), and the other an hypoaneuploid (abnormal) complement (2n=51) comprising 25 pairs of homologous chromosomes plus 1 singleton chromosome (monosomic). In this example, the euploid TM-1 served as a reference genotype, and was

compared to H10, which is monosomic for chromosome-10 (only one copy of that chromosome, but two copies of all other chromosomes, including chromosome-16). In this example of CLA, the relative dosage of the genomic sequence amplified by PCR was gauged by using FAM and HEX fluorescence that correlates to the relative amounts of PCR-based amplification from a primer set that amplifies a chromosome-10 genomic sequence, versus a primer set that amplifies a chromosome-16 genomic sequence. Whereas TM-1 and H10 do NOT differ in dosage for the targeted reference chromosome-16 genomic sequence, they do differ two-fold for the targeted chromosome-10 genomic sequence.

The relative copy number of a chromosome-10 locus in the H10 monosomic stock of *Gossypium hirsutum* L. (one copy of chromosome-10 and two copies of chromosome-16 per cell), was compared to the relative copy number of chromosome-10 in the TM1 control (two copies of chromosome-10 and two copies of chromosome-16 per cell). Chromosome-16 was used as a reference point for both sets of samples, and the ratio of FAM/HEX fluorescence signals from PCR amplification of the chromosome-10 and -16 loci was used to evaluate the H10 and TM-1 genotypes.

Reactions were set up with DNA extracted from leaf tissue from the H10 or TM-1 plants using the NucleoSpin® Plant II Kit (MACHERY-NAGEL GmbH & Co. KG); at least two blanks were used as non-template controls. The reactions contained KASP Master Mix (LGC, Limited), which contains the FAM™-specific cassette (complex of 5' FAM™-labeled oligonucleotide (SEQ ID NO:1) and 3' Quencher labeled oligonucleotide (SEQ ID NO:2)), HEX™-specific cassette (complex of 5' HEX™-labeled oligonucleotide (SEQ ID NO:3) and 3' Quencher labeled oligonucleotide (SEQ ID NO:4)), Taq polymerase, buffer, and passive reference dye ROX™, 60 ng of DNA from either H10 (two different samples, A11 and A12) or TM1, 0.224 μM each of primer pair 2A11 for chromosome 10 (forward primer SEQ ID NO:5 (contains oligonucleotide tail sequence corresponding to FAM™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:6), 0.224 μM each of primer pair 1B04 for chromosome 16 (forward primer SEQ ID NO:7 (contains oligonucleotide tail sequence corresponding to HEX™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:8), and 0.32 mM $MgCl_2$. The reaction was heated to 94°C for 15 minutes, then run for 10 cycles of 94°C for 20 seconds and 65°C for 60 seconds (dropping 0.6°C per cycle), and then run for 26, 28 or 30 cycles (see Table 1) of 94°C for 20 seconds and 57°C for 60 seconds. Fluorescence of the

sample was then determined using a PHERAstar plate reader (scanner) (BMG Labtech; Ortenburg, Germany). Results are summarized in Table 1 and shown in Figure 1. In Table 1, X/N is the luminance ratio of FAM™/ROX™, Y/N is the luminance ratio of HEX™/ROX™, X/Y is the ratio [(X/N)/(median of X/N values)] / [(Y/N)/(median of Y/N values)], Difference From Baseline is X/Y – [0.9 x minimum (X/Y) value for respective set samples (column)], and Ratio to TM1 is [Difference from Baseline for SAMPLE] / [Mean Difference from Baseline for TM-1].

Table 1

Total PCR Cycles	Sample	X/N	Y/N	X/Y	Difference From Baseline	Ratio to TM1
36	H10-A11	0.234983	0.212911	0.866041	0.259812	0.447441
36	H10-A11	0.229446	0.206921	0.870112	0.17728	0.305306
36	H10-A11	0.218828	0.19794	0.867497	0.174665	0.300803
40	H10-A12	0.160694	0.113575	1.110232	0.417399	0.718833
36	H10-A12	0.216606	0.180045	0.944039	0.251207	0.432622
38	H10-A12	0.195822	0.154644	0.993637	0.300804	0.518037
36	TM1	0.245906	0.150422	1.282796	0.589963	1.016019
36	TM1	0.273725	0.175354	1.224891	0.532059	0.916297
36	TM1	0.324715	0.19409	1.312796	0.619964	1.067684
Median		0.229446	0.180045			
Minimum				0.866041		

The results confirm a lower (~half) copy number of the chromosome-10 locus in the *Gossypium hirsutum* L. aneuploid H10, relative to the euploid control, TM-1, based on comparisons to a chromosome-16 locus, of which equal numbers of copies were expected in the H10 and TM-1 genotypes.

Example 2

CLA Analysis to Determine Copy Number of Chromosome-10 Sequence Based on Normalization to a Chromosome-20 Sequence in Cotton

In the present example CLA analysis results are shown for two kinds of cotton (*Gossypium hirsutum* L.), one with a normal complement (2n=52) comprising 26 pairs of homologous chromosomes (euploid), and the other an hypoaneuploid (abnormal) complement (2n=51) comprising 25 pairs of homologous chromosomes plus 1 singleton chromosome

(monosomic). In this example, the euploid TM-1 served as a reference genotype, and was compared to H10, which is monosomic for chromosome-10 (only one copy of that chromosome, but two copies of all other chromosomes, including chromosome-20). In this example of CLA, the relative dosage of the genomic sequence amplified by PCR was gauged by using FAM and HEX fluorescence that correlates to the relative amounts of PCR-based amplification from a primer set that amplifies a chromosome-10 genomic sequence, versus a primer set that amplifies a chromosome-20 genomic sequence. Whereas TM-1 and H10 do NOT differ in dosage for the targeted reference chromosome-20 genomic sequence, they do differ two-fold for the targeted chromosome-10 genomic sequence.

The relative copy number of a chromosome-10 locus in the H10 monosomic stock of *Gossypium hirsutum* L. (one copy of chromosome-10 and two copies of chromosome-20 per cell), was compared to the relative copy number of chromosome-10 in the TM1 control (two copies of chromosome-10 and two copies of chromosome-20 per cell). Chromosome-20 was used as a reference point for both sets of samples, and the ratio of FAM/HEX fluorescence signals from PCR amplification of the chromosome-10 and -20 loci was used to evaluate the H10 and TM-1 genotypes.

Reactions were set up with DNA extracted from leaf tissue from the H10 or TM-1 plants using the NucleoSpin® Plant II Kit (MACHERY-NAGEL GmbH & Co. KG); at least two blanks were used as non-template controls. The reactions contained 1x KASP Master Mix (LGC, Limited), which contains the FAM™-specific cassette (complex of 5' FAM-labeled oligonucleotide (SEQ ID NO:1) and 3' Quencher labeled oligonucleotide (SEQ ID NO:2)), HEX™-specific cassette (complex of 5' HEX-labeled oligonucleotide (SEQ ID NO:3) and 3' Quencher labeled oligonucleotide (SEQ ID NO:4)), Taq polymerase, buffer, and passive reference dye ROX™, 60 ng of DNA from either H10 (two different samples, A11 and A12) or TM1, 0.224 µM each of primer pair 2E04 for chromosome 10 (forward primer SEQ ID NO:9 (contains oligonucleotide tail sequence corresponding to HEX™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:10), 0.224 µM each of primer pair 2A03 for chromosome 20 (forward primer SEQ ID NO:11 (contains oligonucleotide tail sequence corresponding to FAM™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:12), and 0.32 mM $MgCl_2$. The reaction was heated to 94°C for 15 minutes, then run for 10 cycles of 94°C for 20 seconds and 65°C for 60 seconds (dropping 0.6°C per cycle), and then run for 28 or

30 cycles (see Table 2) of 94°C for 20 seconds and 57°C for 60 seconds. Fluorescence of the sample was then determined using a PHERAstar plate reader (scanner) (BMG Labtech; Ortenburg, Germany). Results are summarized in Table 2 and shown in Figure 2. In Table 2, X/N is the luminance ratio of FAM™/ROX™, Y/N is the luminance ratio of HEX™/ROX™, X/Y is the ratio [(X/N)/(median of X/N values)] / [(Y/N)/(median of Y/N values)], Difference From Baseline is X/Y – [0.9 x minimum (X/Y) value for respective set samples (column)], and Ratio to TM1 is [Difference from Baseline for SAMPLE] / [Mean Difference from Baseline for TM-1].

Table 2

Total PCR Cycles	Sample	X/N	Y/N	X/Y	Difference From Baseline	Ratio to TM1
38	H10-A11	0.81043	0.271365	1.210766	0.503579	2.403852
38	H10-A11	1.127366	0.387271	1.180182	0.472995	2.257859
38	H10-A11	1.17658	0.407728	1.169904	0.462717	2.208796
38	H10-A12	0.770271	0.263336	1.185857	0.47867	2.284949
38	H10-A12	0.951358	0.330334	1.167588	0.460401	2.197743
38	H10-A12	0.991906	0.34174	1.176721	0.469534	2.241339
40	TM1	0.930847	0.426906	0.883984	0.176797	0.843946
38	TM1	0.955247	0.422861	0.915833	0.208646	0.995981
38	TM1	1.08257	0.461887	0.950209	0.243022	1.160074
Median		0.955247	0.387271			
Minimum				0.883984		

The results confirm a lower (~half) copy number of the chromosome-10 locus in the *Gossypium hirsutum* L. aneuploid H10, relative to the euploid control, TM-1, based on comparisons to a chromosome-20 locus, of which equal numbers of copies were expected in the H10 and TM-1 genotypes.

Example 3

CLA Analysis to Determine Copy Number of Chromosome-11 Sequence Based on Normalization to a Chromosome-20 Sequence in Cotton

In the present example CLA analysis results are shown for two kinds of cotton (*Gossypium hirsutum* L.), one with a normal complement (2n=52) comprising 26 pairs of homologous chromosomes (euploid), and the other an hypoaneuploid (abnormal) complement

(2n=51) comprising 25 pairs of homologous chromosomes plus 1 singleton chromosome (monosomic). In this example, the euploid TM-1 served as a reference genotype, and was compared to H11, which is monosomic for chromosome-11 (only one copy of that chromosome, but two copies of all other chromosomes, including chromosome-20). In this example of CLA, the relative dosage of the genomic sequence amplified by PCR was gauged by using FAM and HEX fluorescence that correlates to the relative amounts of PCR-based amplification from a primer set that amplifies a chromosome-11 genomic sequence, versus a primer set that amplifies a chromosome-20 genomic sequence. Whereas TM-1 and H11 do NOT differ in dosage for the targeted reference chromosome-20 genomic sequence, they do differ two-fold for the targeted chromosome-11 genomic sequence.

The relative copy number of a chromosome-11 locus in the H11 monosomic stock of *Gossypium hirsutum* L. (one copy of chromosome-11 and two copies of chromosome-20 per cell), was compared to the relative copy number of chromosome-11 in the TM1 control (two copies of chromosome-11 and two copies of chromosome-20 per cell). Chromosome-20 was used as a reference point for both sets of samples, and the ratio of FAM/HEX fluorescence signals from PCR amplification of the chromosome-11 and -20 loci was used to evaluate the H11 and TM-1 genotypes.

Reactions were set up with DNA extracted from leaf tissue from the H11 or TM-1 plants using the NucleoSpin® Plant II Kit (MACHERY-NAGEL GmbH & Co. KG); at least two blanks were used as non-template controls. The reactions contained 1x KASP Master Mix (LGC, Limited), which contains the FAM™-specific cassette (complex of 5' FAM-labeled oligonucleotide (SEQ ID NO:1) and 3' Quencher labeled oligonucleotide (SEQ ID NO:2)), HEX™-specific cassette (complex of 5' HEX-labeled oligonucleotide (SEQ ID NO:3) and 3' Quencher labeled oligonucleotide (SEQ ID NO:4)), Taq polymerase, buffer, and passive reference dye ROX™, 60 ng of DNA from either H11 (five different samples, B01, B02, B03, B04 and B05) or TM1, 0.224 μM each of primer pair 3G02 for chromosome 11 (forward primer SEQ ID NO:13 (contains oligonucleotide tail sequence corresponding to HEX™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:14), 0.224 μM each of primer pair 2C11 for chromosome 20 (forward primer SEQ ID NO:15 (contains oligonucleotide tail sequence corresponding to FAM™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:16), and 0.32 mM $MgCl_2$. The reaction was heated to 94°C for 15 minutes, then run for

10 cycles of 94°C for 20 seconds and 65°C for 60 seconds (dropping 0.6°C per cycle), and then run for 28 or 30 cycles (see Table 3) of 94°C for 20 seconds and 57°C for 60 seconds. Fluorescence of the sample was then determined using a PHERAstar plate reader (scanner) (BMG Labtech; Ortenburg, Germany). Results are summarized in Table 3 and shown in Figure 3. In Table 3, X/N is the luminance ratio of FAM™/ROX™, Y/N is the luminance ratio of HEX™/ROX™, X/Y is the ratio [(X/N)/(median of X/N values)] / [(Y/N)/(median of Y/N values)], Difference From Baseline is X/Y – [0.9 x minimum (X/Y) value for respective set samples (column)], and Ratio to TM1 is [Difference from Baseline for SAMPLE] / [Mean Difference from Baseline for TM-1].

Table 3

Total PCR Cycles	Sample	X/N	Y/N	X/Y	Difference From Baseline	Ratio to TM1
40	H11-B01	0.784798	0.33255	1.043379	0.369525	2.093059
40	H11-B01	0.925395	0.404428	1.011643	0.337789	1.913299
40	H11-B01	0.996444	0.436139	1.010111	0.336257	1.904622
40	H11-B02	0.874897	0.364144	1.062246	0.388392	2.199924
40	H11-B02	0.90913	0.395971	1.015089	0.341235	1.932818
40	H11-B02	0.961566	0.417609	1.018006	0.344153	1.949343
40	H11-B03	0.796294	0.339425	1.03722	0.363367	2.058175
40	H11-B03	0.899646	0.389109	1.022214	0.348361	1.973179
40	H11-B03	0.918225	0.411769	0.985909	0.312056	1.76754
40	H11-B04	0.821619	0.36244	1.00225	0.328396	1.860097
40	H11-B04	0.895938	0.390315	1.014855	0.341002	1.931497
40	H11-B04	0.931873	0.399882	1.030307	0.356454	2.019019
40	H11-B05	0.89839	0.373004	1.06486	0.391006	2.214732
40	H11-B05	0.933251	0.401323	1.028125	0.354272	2.006659
40	H11-B05	0.973838	0.423621	1.016368	0.342514	1.940065
40	TM1	0.802758	0.41388	0.857535	0.183681	1.040404
40	TM1	0.768278	0.398979	0.851353	0.177499	1.005389
38	TM1	0.68989	0.362114	0.842317	0.168463	0.954208
Median		0.899018	0.397475			
Minimum				0.842317		

The results confirm a lower (~half) copy number of the chromosome-11 locus in the *Gossypium hirsutum* L. aneuploid H11, relative to the euploid control, TM-1, based on

comparisons to a chromosome-20 locus, of which equal numbers of copies were expected in the H11 and TM-1 genotypes.

Example 4

CLA Analysis to Determine Copy Number of Chromosome-20 Sequence Based on Normalization to a Chromosome-10 Sequence in Cotton

In the present example CLA analysis results are shown for two kinds of cotton (*Gossypium hirsutum* L.), one with a normal complement (2n=52) comprising 26 pairs of homologous chromosomes (euploid), and the other an hypoaneuploid (abnormal) complement (2n=51) comprising 25 pairs of homologous chromosomes plus 1 singleton chromosome (monosomic). In this example, the euploid TM-1 served as a reference genotype, and was compared to H20, which is monosomic for chromosome-20 (only one copy of that chromosome, but two copies of all other chromosomes, including chromosome-10). In this example of CLA, the relative dosage of the genomic sequence amplified by PCR was gauged by using FAM and HEX fluorescence that correlates to the relative amounts of PCR-based amplification from a primer set that amplifies a chromosome-20 genomic sequence, versus a primer set that amplifies a chromosome-10 genomic sequence. Whereas TM-1 and H20 do NOT differ in dosage for the targeted reference chromosome-10 genomic sequence, they do differ two-fold for the targeted chromosome-20 genomic sequence.

The relative copy number of a chromosome-20 locus in the H20 monosomic stock of *Gossypium hirsutum* L. (one copy of chromosome-20 and two copies of chromosome-10 per cell), was compared to the relative copy number of chromosome-20 in the TM1 control (two copies of chromosome-20 and two copies of chromosome-10 per cell). Chromosome-10 was used as a reference point for both sets of samples, and the ratio of FAM/HEX fluorescence signals from PCR amplification of the chromosome-20 and -10 loci was used to evaluate the H20 and TM-1 genotypes.

Reactions were set up with DNA extracted from leaf tissue from the H20 or TM-1 plants using the NucleoSpin® Plant II Kit (MACHERY-NAGEL GmbH & Co. KG); at least two blanks were used as non-template controls. The reactions contained 1x KASP Master Mix (LGC, Limited), which contains the FAM™-specific cassette (complex of 5' FAM™-labeled oligonucleotide (SEQ ID NO:1) and 3' Quencher labeled oligonucleotide (SEQ ID NO:2)),

HEX™-specific cassette (complex of 5' HEX™-labeled oligonucleotide (SEQ ID NO:3) and 3' Quencher labeled oligonucleotide (SEQ ID NO:4)), Taq polymerase, buffer, and passive reference dye ROX™, 60 ng of DNA from either H20 (four different samples, 1408012.05, 1408012.06, 1408012.07, and 1408012.08) or TM1, 0.224 µM each of primer pair 2A03 for chromosome 20 (forward primer SEQ ID NO:11 (contains oligonucleotide tail sequence corresponding to FAM™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:12), 0.224 µM each of primer pair 2E04 for chromosome 10 (forward primer SEQ ID NO:9 (contains oligonucleotide tail sequence corresponding to HEX™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:10), and 0.32 mM $MgCl_2$. The reaction was heated to 94°C for 15 minutes, then run for 10 cycles of 94°C for 20 seconds and 65°C for 60 seconds (dropping 0.6°C per cycle), and then run for 26 or 30 cycles (see Table 4) of 94°C for 20 seconds and 57°C for 60 seconds. Fluorescence of the sample was then determined using a PHERAstar plate reader (scanner) (BMG Labtech; Ortenburg, Germany). Results are summarized in Table 4 and shown in Figure 4. In Table 4, X/N is the luminance ratio of FAM™/ROX™, Y/N is the luminance ratio of HEX™/ROX™, X/Y is the ratio [(X/N)/(median of X/N values)] / [(Y/N)/(median of Y/N values)], Difference From Baseline is X/Y – [0.9 x minimum (X/Y) value for respective set samples (column)], and Ratio to TM1 is [Difference from Baseline for SAMPLE] / [Mean Difference from Baseline for TM-1].

Table 4

Total PCR Cycles	Sample	X/N	Y/N	X/Y	Difference From Baseline	Ratio to TM1
36	1408012.05	0.561164	0.346618	0.897422427	0.209336	0.427809
40	1408012.05	0.69145	0.421945	0.908370294	0.220283	0.450183
36	1408012.06	0.660094	0.382072	0.957677881	0.269591	0.55095
40	1408012.06	0.667299	0.401951	0.920249595	0.232163	0.47446
36	1408012.07	0.511859	0.32988	0.860108645	0.172022	0.351553
40	1408012.07	0.548568	0.35343	0.86036989	0.172283	0.352087
36	1408012.08	0.565164	0.353726	0.885658771	0.197572	0.403768
40	1408012.08	0.819901	0.459462	0.989169525	0.301083	0.615308
36	TM1	0.72029	0.336468	1.18665046	0.498564	1.018891
40	TM1	0.928919	0.440791	1.168163278	0.480076	0.981109
Median		0.663697	0.367899			
Minimum				0.860108645		

The results confirm a lower (~half) copy number of the chromosome-20 locus in the *Gossypium hirsutum* L. aneuploid H20, relative to the euploid control, TM-1, based on comparisons to a chromosome-10 locus, of which equal numbers of copies were expected in the H20 and TM-1 genotypes.

Example 5

CLA Analysis to Determine Copy Number of Chromosome-20 Sequence Based on Normalization to a Chromosome-11 Sequence in Cotton

In the present example CLA analysis results are shown for two kinds of cotton (*Gossypium hirsutum* L.), one with a normal complement (2n=52) comprising 26 pairs of homologous chromosomes (euploid), and the other an hypoaneuploid (abnormal) complement (2n=51) comprising 25 pairs of homologous chromosomes plus 1 singleton chromosome (monosomic). In this example, the euploid TM-1 served as a reference genotype, and was compared to H20, which is monosomic for chromosome-20 (only one copy of that chromosome, but two copies of all other chromosomes, including chromosome-11). In this example of CLA, the relative dosage of the genomic sequence amplified by PCR was gauged by using FAM and HEX fluorescence that correlates to the relative amounts of PCR-based amplification from a primer set that amplifies a chromosome-20 genomic sequence, versus a primer set that amplifies a chromosome-11 genomic sequence. Whereas TM-1 and H20 do NOT differ in dosage for the targeted reference chromosome-11 genomic sequence, they do differ two-fold for the targeted chromosome-20 genomic sequence.

The relative copy number of a chromosome-20 locus in the H20 monosomic stock of *Gossypium hirsutum* L. (one copy of chromosome-20 and two copies of chromosome-11 per cell), was compared to the relative copy number of chromosome-20 in the TM1 control (two copies of chromosome-20 and two copies of chromosome-11 per cell). Chromosome-11 was used as a reference point for both sets of samples, and the ratio of FAM/HEX fluorescence signals from PCR amplification of the chromosome-20 and -11 loci was used to evaluate the H20 and TM-1 genotypes.

Reactions were set up with DNA extracted from leaf tissue from the H20 or TM-1 plants using the NucleoSpin® Plant II Kit (MACHERY-NAGEL GmbH & Co. KG); at least two blanks were used as non-template controls. The reactions contained 1x KASP Master Mix

(LGC, Limited), which contains the FAM™-specific cassette (complex of 5' FAM™-labeled oligonucleotide (SEQ ID NO:1) and 3' Quencher labeled oligonucleotide (SEQ ID NO:2)), HEX™-specific cassette (complex of 5' HEX™-labeled oligonucleotide (SEQ ID NO:3) and 3' Quencher labeled oligonucleotide (SEQ ID NO:4)), Taq polymerase, buffer, and passive reference dye ROX™, 60 ng of DNA from either H20 (four different samples, 1408012.05, 1408012.06, 1408012.07, and 1408012.08) or TM1, 0.224 µM each of primer pair 2C11 for chromosome 20 (forward primer SEQ ID NO:15 (contains oligonucleotide tail sequence corresponding to FAM™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:16), 0.224 µM each of primer pair 3G02 for chromosome 11 (forward primer SEQ ID NO:13 (contains oligonucleotide tail sequence corresponding to HEX™-labeled oligonucleotide sequence) and reverse primer SEQ ID NO:14), and 0.32 mM $MgCl_2$. The reaction was heated to 94°C for 15 minutes, then run for 10 cycles of 94°C for 20 seconds and 65°C for 60 seconds (dropping 0.6°C per cycle), and then run for 26 or 28 cycles (see Table 5) of 94°C for 20 seconds and 57°C for 60 seconds. Fluorescence of the sample was then determined using a PHERAstar plate reader (scanner) (BMG Labtech; Ortenburg, Germany). Results are summarized in Table 5 and shown in Figure 5. In Table 5, X/N is the luminance ratio of FAM™/ROX™, Y/N is the luminance ratio of HEX™/ROX™, X/Y is the ratio [(X/N)/(median of X/N values)] / [(Y/N)/(median of Y/N values)], Difference From Baseline is X/Y – [0.9 x minimum (X/Y) value for respective set samples (column)], and Ratio to TM1 is [Difference from Baseline for SAMPLE] / [Mean Difference from Baseline for TM-1].

Table 5

Total PCR Cycles	Sample	X/N	Y/N	X/Y	Difference From Base.	Ratio to TM1
36	1408012.05	0.76845	0.376612	0.942146817	0.107196	0.416904
38	1408012.05	0.785636	0.370644	0.97872862	0.143778	0.559177
36	1408012.06	0.755303	0.368788	0.945675623	0.110725	0.430628
38	1408012.06	0.803514	0.374277	0.991282332	0.156332	0.608
36	1408012.07	0.692775	0.344803	0.927722812	0.092772	0.360807
38	1408012.07	0.771047	0.347494	1.024546094	0.189596	0.737368
36	1408012.08	0.719843	0.320647	1.03659236	0.201642	0.784218
38	1408012.08	0.783159	0.36543	0.989562329	0.154612	0.601311
36	TM1	0.817299	0.352209	1.071465428	0.236515	0.919845
38	TM1	0.824828	0.342286	1.112684921	0.277734	1.080155
Median		0.777103	0.35882			
Minimum				0.927722812		

The results confirm a lower (~half) copy number of the chromosome-20 locus in the *Gossypium hirsutum* L. aneuploid H20, relative to the euploid control, TM-1, based on comparisons to a chromosome-11 locus, of which equal numbers of copies were expected in the H20 and TM-1 genotypes.

Example 6

CLA Analysis Using Standardized Reference Primer Set

In the present example CLA analysis results are shown using a standardized reference primer set common to all assays, and the use of gBlocks® to synthesize the reference standards. Previous assays were run using a variety of target primers that were designed for different assays as a reference. Adopting a standard reference primer set greatly simplified the process. Ubiquitin-conjugating enzyme (UBC1) is present in a single-copy per haploid cotton (*Gossypium hirsutum* L.) genome, making it a suitable standardized reference (Yi, *et al.*, *Analytical Biochem.* 375:150-152, 2008). In addition, the SAD1 gene can be used as reference in cotton (Yang, *et al.*, *Plant Cell Rep.* 24:237-245, 2005). A pair of DNA primers (forward and reverse) were designed for targeted PCR-based amplification of a small genomic DNA sequence that is contained within or equates to the borders of a known, putative or possible copy number variant. To the designed sequence of at least one of the pair of primers, a DNA sequence "Tag" was added corresponding to the FAM/X fluorochrome labeled DNA sequence in the KASP master-mix (KASP MM), such that PCR-based amplification of sample DNA using the "tagged" primer(s) leads to incorporation of the tagged sequence into the PCR product (amplicon) and de-quenching of the FAM fluorochrome, resulting in FAM fluorescence. The primers were designed for sequences within UBC1, and tested them to identify combination(s) of forward and reverse primers that consistently yielded good amplification of the target sequence and no self-amplification. These were labeled with a sequence corresponding to the HEX/Y fluorochrome in the master-mix.

In the above design, FAM and HEX are to be used to detect amplification of the Target (FAM signal) versus Reference (HEX signal); these roles can be easily reversed by swapping the use of their respective DNA sequence "Tags" in the locus-specific primers (Target and Reference). While the commercial availability of KASP MM facilitates implementation of these assays, successful implementation of these methods of CNV detection is not relegated to the

specific tag DNA sequences or tag fluorochromes available in commercially vended KASP MM. Whereas SNP detection and genotyping using KASP assays and KASP MM rely on the two allele-specific forward primers being "tagged" with fluorochrome-specifying KASP MM oligo-specific DNA sequences and the common reverse primer being non-tagged, the CLA implementation can utilize fluorochrome-specific tagging of the forward and/or reverse allele-specific primers. Thus the CLA assay, *e.g.*, for detection of relatively large CN differences or presence or copy number of transgene sequences, is more flexible than the KASP assay commonly applied to SNP detection.

The reaction included: 0.712 μL H_20, 4.0 μL KASP Master Mix, 0.112 μL target primer pair, 0.112 μL reference primer pair, 0.064 μL 100 mM MgCl, and 3 μL DNA template diluted in nanopure water to 10 ng/μL. Thermocycler conditions: 95°C for 15 minutes, 10 cycles of 95°C (20 seconds)/61°C (60 seconds) dropping 0.6°C every cycle to 55°C, 14 cycles of 95°C (20 seconds)/55°C (60 seconds). At this point it is scanned (24 cycles). Then a single cycle of 95°C (20 seconds)/55°C (60 seconds) is added and scanned. This is repeated for 40-50 total cycles. It certain aspects, it is sufficient and more practical to scan intermittently, *e.g.*, every third or fifth cycle, and to initiate the scans at a cycle other than the 24th. Fluorescence data is measured at each cycle for each well. Optimal cycling conditions are determined experimentally as these vary with different primer sets, sample quality, minor variations in starting material, *etc.* Typically, data collection begins at 24 cycles and ends at 40 cycles. This consists of three measurements: FAM or X (485 nm) corresponding to target primer amplification; HEX or Y (535 nm) corresponding to reference primer amplification; and ROX or N (575 nm) a passive reference dye for normalizing expression between wells. The normalized value for X and Y is that value divided by the fluorescence for ROX (X/N and Y/N).

A threshold value is set in relation to a no template control (NTC), a reaction mixture in which ddH_20 has been added to the reaction components in place of a DNA template. Any expression at or below the NTC indicates amplification has not yet occurred at a measurable level, and such a signal is considered mere noise. Typically, the threshold is set to be 1.1-1.4 times the NTC value for each dye, a value that is experimentally determined. Useful data can be obtained from samples during cycles where the level of expression is consistently above the NTC and steadily increases (amplifies) from one cycle to another. In practice, it is possible for a well to fluoresce at a somewhat higher rate than the NTC in one cycle then be well below in the

subsequence cycle; this is not considered to be above threshold and is merely due to variability in the scan. Additionally, a maximum ceiling is set, typically 4-5 times the NTC value, where expression levels seem to "max out" and there are little to no increases in fluorescence with additional cycles. Once expression for both FAM and HEX have surpassed this threshold the fluorescence emission ratio is recorded as Xn/Yn for each well and for each cycle where they are above threshold and below ceiling. The median of these ratios is then recorded as the average expression ratio. This is then baselined relative to other samples in the assay. This means that some portion of the lower end of the ratio is removed to focus on the meaningful differences between them. This is typically between 50-80% of the lowest ratio in that set. For example, if the ratio is 1.6 and 1.1 for the euploid and aneuploid, respectively, the bottom 0.6 might be removed from both values, so the ratio becomes 1.0 to 0.5. These baselined ratios are then set relative to a known euploid *G. hirsutum* control, line TM1 typically, which will always be 1.0. If the target is present in lower quantities the relative expression ratio for that sample will be lower than 1.0; higher quantities will be greater than one.

In addition gBlocks® (Integrated DNA Technologies, Inc., Coralville, IA) were used to synthesize dosage-reference standards. These are short (~500 bp) custom-made double-stranded DNA sequences that are used to establish a synthetic analytical platform that significantly facilitates testing and quantitatively assessing the fundamental ability of primer sets to discern essentially any range of CNV, without the need for the presence of a known aneuploid or biological variants. To create a synthetic target "gBlock" multiple DNA sequences corresponding to the target sequence amplified by the target primers were joined together to form a single 500 bp double-stranded synthetic DNA sequence. A separate "gBlock" corresponding to the universal reference sequences (UBC1 and SAD1) amplified by the reference primers were also created. Multiple reference templates were created by combining the target and reference "gBlock" sequences at varying ratios (*e.g.*, 1:1, 1:2, 1:4, *etc.*) to show the ability of this protocol to differentiate different copy numbers. These synthetic references can then be used as standards when looking for CNV in unknown samples in the absence of a known CN reference.

Results of a dilution series test for using one target primer set (target is chromosome 11, the reference is UBC1) is shown in FIG. 6. The normalization protocol (*i.e.*, thresholds and baseline) was established to optimize the fit between the experimentally observed and expected (line). Using this normalization protocol, the corresponding euploid TM1 genetic/cytogenetic

standard and chromosome-11 monosomic aneuploids ("H11") were analyzed to determine where they fell relative to the synthetic gBlock dilutions; for example, the signal ratio from H11 versus TM1 plants for unique sequences in chromosome-11 would be expected to be similar to that of "gBlock" ratio 1:2). The results are shown in FIG. 7. The bottom line indicates the median value for the 1:2 "gBlock" dilution. The H11 samples represent three chromosome-11 monosomic aneuploids ("H11") assayed. They all fall closer to the 1:2 "gBlock" dilution than to either the 1:4 or 3:4. So this indicates a successful assay for this target. TM1 was also assessed and aligned with the 1:1 control "gBlock" as expected.

To test sensitivity of FAM/HEX signal ratios to starting concentration of sample template DNA (biological gDNA), a dilution series of various starting quantities (10 ng-80 ng of *G. hirsutum* TM-1 DNA) was run. The concentration of a sample of the DNA was adjusted to 20 ng/µl, based on DeNovix® DS-11 fluorometric analysis, and that 20 ng/µl sample was then diluted and quantified to create a range of concentrations (10, 5 and 2.5 ng/µl). The results are shown in FIG. 8. The variability increases in lower starting concentrations, but it is still possible to discern the difference between a 1:2 copy number (CN) difference within this range (p-value = 1.0e-13 in a two-tailed t-test, unequal variance). A wide range of viable starting concentrations that will not require the use of precise (pico-green) standardization greatly streamlines the process and significantly reduces the per sample costs.

The results of various tests against known aneuploids are provided (FIG. 9 to FIG. 15). The Y-axis is normalized relative fluorescence. It is normalized based on the threshold value described above. It is then set relative to the control (TM1) such that the median value for the TM1 fluorescence will always be 1.0. All individuals were assessed in triplicate using as many individuals as were available for that particular aneuploid family. The horizontal bottom line corresponds to the median value of the aneuploids assessed; the horizontal top line corresponds to the median for TM1 (1.0). The universal reference used was UBC1, except for FIG. 13, which used SAD1 as the universal reference. Target primers correspond to a sequenced region of the chromosome listed in the title. The x-axis are sample names: "H#" indicates the aneuploid line with # indicating the monoploid chromosome. The TM1 standard is always given last, on the far right as "TM1," a euploid standard of cotton (*Gossypium hirsutum* L.). Some assay exhibit greater variability than others, but this could be due to human error. However, all assays

presented were able to discern the CN variant (aneuploid) from the standard (two-tail t-test unequal variance: $p < 0.01$).

Example 7

CLA Analysis to Determine Copy Number of Transgenic Insert

One application of the presently described CLA analysis is to screen transgenic or gene-edited (*e.g.*, with single-base changes) germplasm for CN of the alien insert. While it is easy to test for the presence/absence of transgenic material using conventional PCR, it is much more difficult to discern individuals with a single copy of the transgenic sequence from those with multiple copies or fragments. It is relatively expensive to use commercially vended test strips, and these may not be applicable to newly created transgenes or gene-edited products. DNA was acquired from two transformed lines; one contains a single copy of the transgene, the other contains a single complete copy plus an additional fragment. For a control, these were analyzed relative to the *G. hirsutum* line (Coker 312) from which they were derived. FIG. 16 and FIG. 17 show the results of a control when the alien insert is present in the same copy number for both transformed lines, FIG. 18 and FIG. 19 show the results of a control for the alien insert with copy number differences between the transformed lines due to the presence of an insert fragment, and FIG. 20 and FIG. 21 show the results of the *Gossypium* insert with a copy number difference between transformed lines and the Coker control.

* * * * * * * * * * * * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

Claim 1. A method of determining copy number of a nucleotide sequence of interest in a nucleic acid sample, comprising:

a) amplifying the nucleotide sequence of interest or a portion thereof in the nucleic acid sample using a first oligonucleotide primer comprising a 3' region that specifically binds to said nucleotide sequence of interest or portion thereof and a 5' region that comprises a nucleotide sequence corresponding to a first fluorescent-labeled oligonucleotide primer and a second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the nucleic acid sample using a third oligonucleotide primer comprising a 3' region that specifically binds to the control nucleotide sequence and a 5' region that comprises a nucleotide sequence corresponding to a second fluorescent-labeled oligonucleotide primer and a fourth oligonucleotide primer, in a reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer; and

b) comparing fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer with fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, to determine the copy number of the nucleotide sequence of interest.

Claim 2. The method of claim 1, wherein the nucleotide sequence of interest comprises genomic DNA.

Claim 3. The method of claim 2, wherein the nucleotide sequence of interest is a gene or regulatory element.

Claim 4. The method of claim 3, wherein the nucleotide sequence of interest is a gene.

Claim 5. The method of claim 1, wherein the nucleic acid sample is from a plant.

Claim 6. The method of claim 5, wherein the nucleic acid sample is from a cotton, sorghum, maize, tomato, rice, barley or wheat plant.

Claim 7. The method of claim 6, wherein the nucleic acid sample is from a cotton plant.

Claim 8. The method of claim 1, wherein the nucleic acid sample is from a mammal.

Claim 9. The method of claim 8, wherein the mammal is a livestock animal.

Claim 10. The method of claim 9, wherein the livestock animal is a cow, pig, sheep, chicken, goat, horse, turkey, duck, or goose.

Claim 11. The method of claim 8, wherein the nucleic acid sample is from a human.

Claim 12. The method of claim 1, wherein the first fluorescent-labeled oligonucleotide primer is labeled with fluorescein, VIC®, 6-fluorescein, hexachloro-fluorescein, carboxy-tetramethyl-rhodamine, carboxy-X-rhodamine, or 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine.

Claim 13. The method of claim 12, wherein the first fluorescent-labeled oligonucleotide primer is labeled with fluorescein.

Claim 14. The method of claim 1, wherein the second fluorescent-labeled oligonucleotide primer is labeled with fluorescein, VIC®, 6-fluorescein, hexachloro-fluorescein, carboxy-

tetramethyl-rhodamine, carboxy-x-rhodamine, or 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine.

Claim 15. The method of claim 14, wherein the second fluorescent-labeled oligonucleotide primer is labeled with hexachloro-fluorescein.

Claim 16. The method of claim 1, wherein the first quenching oligonucleotide primer or the second quenching oligonucleotide primer is labeled with a black hole quencher dye or an ATTO quencher.

Claim 17. The method of claim 16, wherein the first quenching oligonucleotide primer or the second quenching oligonucleotide primer is labeled with a black hole quencher dye.

Claim 18. The method of claim 17, wherein the first quenching oligonucleotide primer and the second quenching oligonucleotide primer is labeled with a black hole quencher dye.

Claim 19. The method of claim 1, further comprising a passive fluorescent marker.

Claim 20. A method of determining copy number of a nucleotide sequence of interest in a first nucleic acid sample, comprising:

a) amplifying the nucleotide sequence of interest or a portion thereof in the first nucleic acid sample using a first oligonucleotide primer comprising a 3' region that specifically binds to said nucleotide sequence of interest or portion thereof and a 5' region that comprises a nucleotide sequence corresponding to a first fluorescent-labeled oligonucleotide primer and a second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the first nucleic acid sample using a third oligonucleotide primer comprising a 3' region that specifically binds to the control nucleotide sequence and a 5' region that comprises a nucleotide sequence corresponding to a second fluorescent-labeled

oligonucleotide primer and a fourth oligonucleotide primer, in a first reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer;

b) amplifying the nucleotide sequence of interest or a portion thereof in a second nucleic acid sample using the first oligonucleotide primer and the second oligonucleotide primer, and amplifying a control nucleotide sequence comprising a known copy number in the second nucleic acid sample using the third oligonucleotide primer and the fourth oligonucleotide primer, in a second reaction comprising the first fluorescent-labeled oligonucleotide primer bound to a first quenching oligonucleotide primer and the second fluorescent-labeled oligonucleotide primer bound to a second quenching oligonucleotide primer;

c) determining a first normalized fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer and a second normalized fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, utilizing fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer and fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer in the first and second reactions; and

d) comparing the first normalized fluorescence of the amplification product comprising the first fluorescent-labeled oligonucleotide primer with the second normalized fluorescence of the amplification product comprising the second fluorescent-labeled oligonucleotide primer, to determine the copy number of the nucleotide sequence of interest.

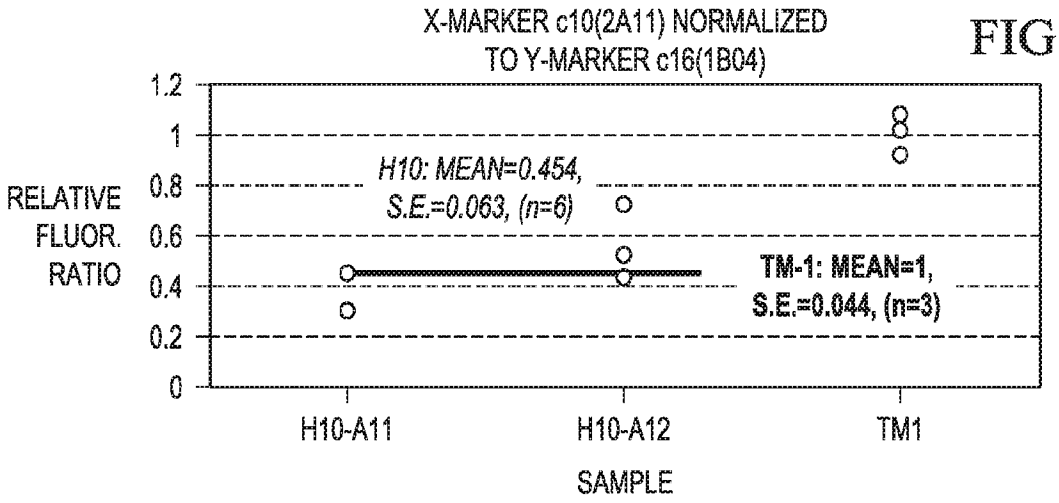


FIG. 1

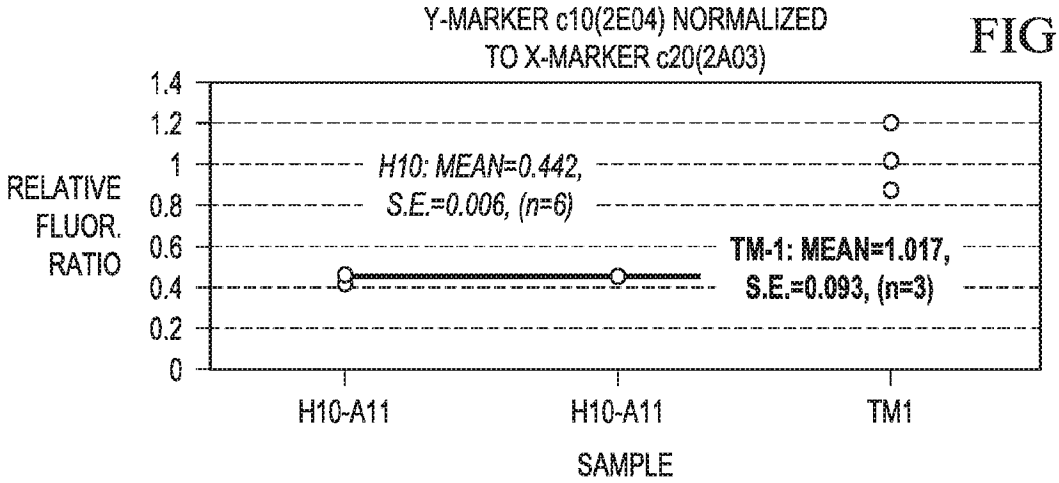


FIG. 2

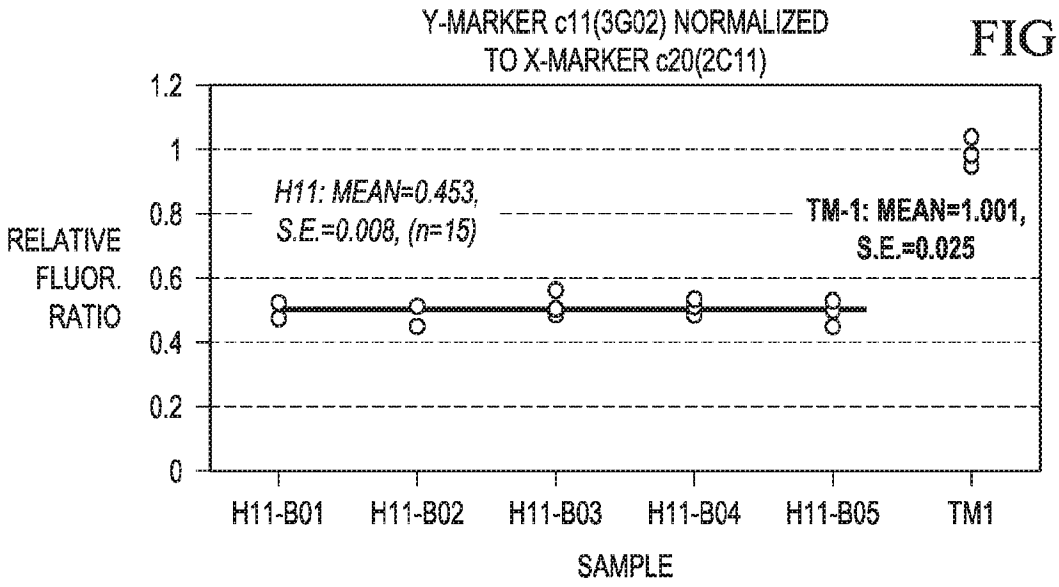


FIG. 3

FIG. 4

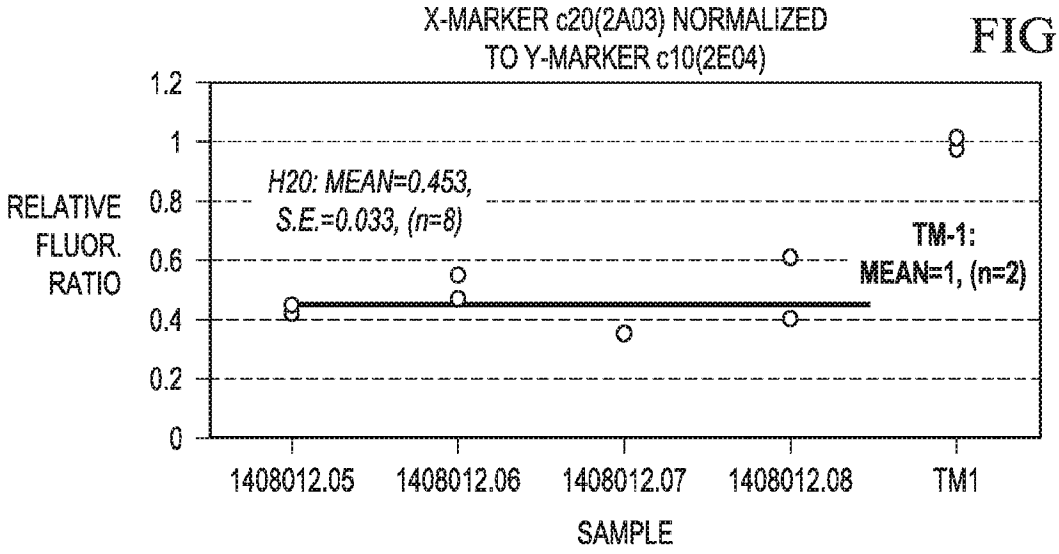


FIG. 5

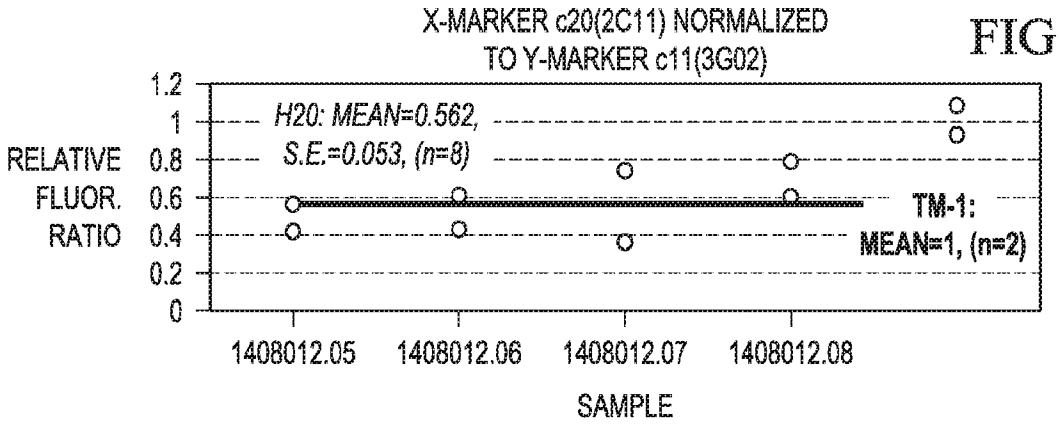
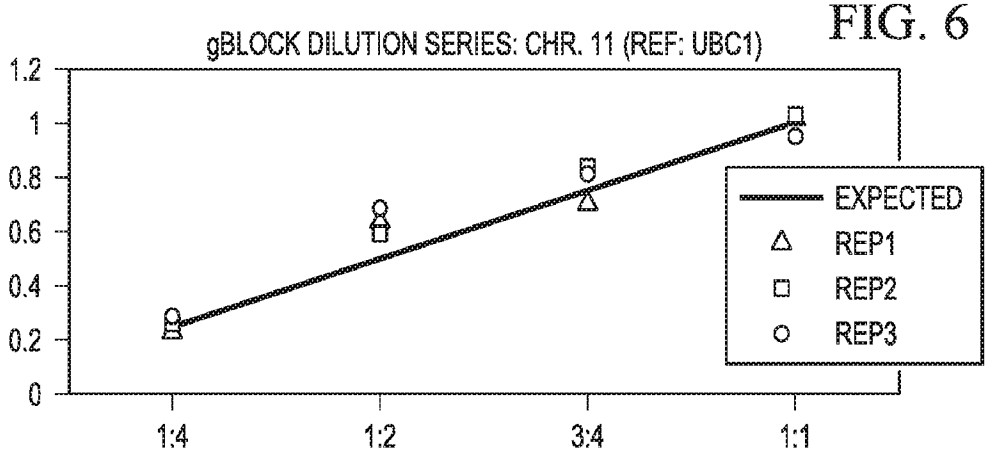


FIG. 6



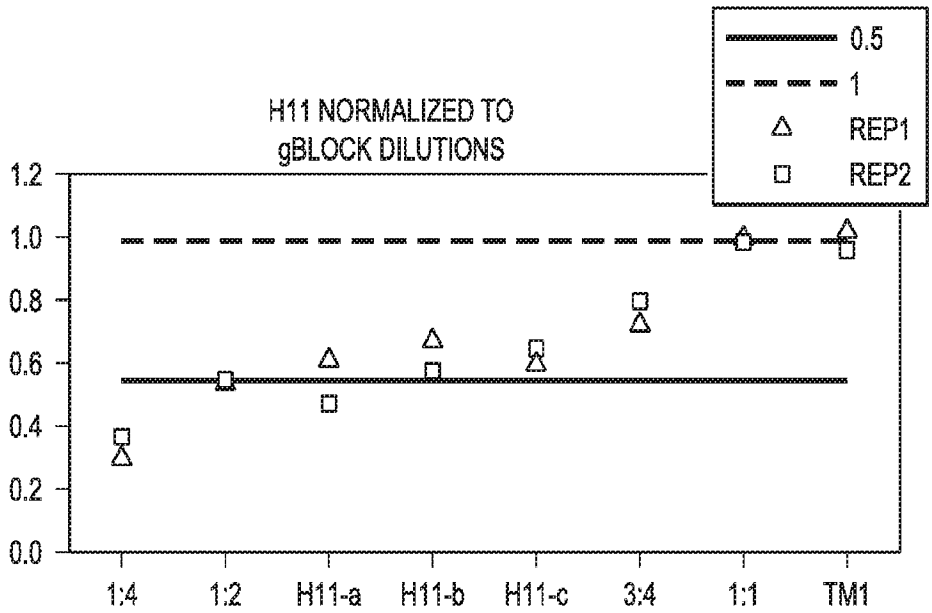

H11 NORMALIZED TO
gBLOCK DILUTIONS
0.5
1
REP1
REP2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1:4
1:2
H11-a
H11-b
H11-c
3:4
1:1
TM1

FIG. 7

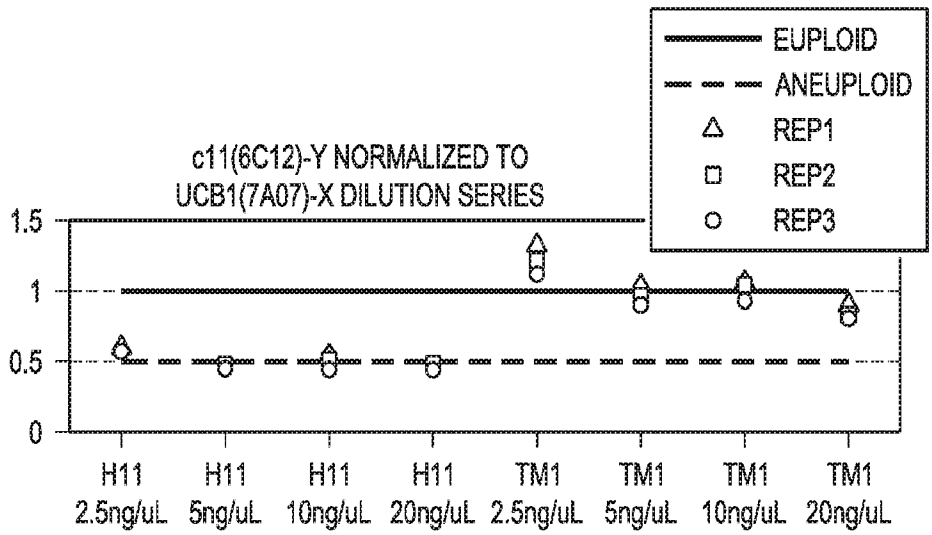

c11(6C12)-Y NORMALIZED TO
UCB1(7A07)-X DILUTION SERIES
EUPLOID
ANEUPLOID
REP1
REP2
REP3
1.5
1
0.5
0
H11
2.5ng/uL
H11
5ng/uL
H11
10ng/uL
H11
20ng/uL
TM1
2.5ng/uL
TM1
5ng/uL
TM1
10ng/uL
TM1
20ng/uL

FIG. 8

FIG. 9

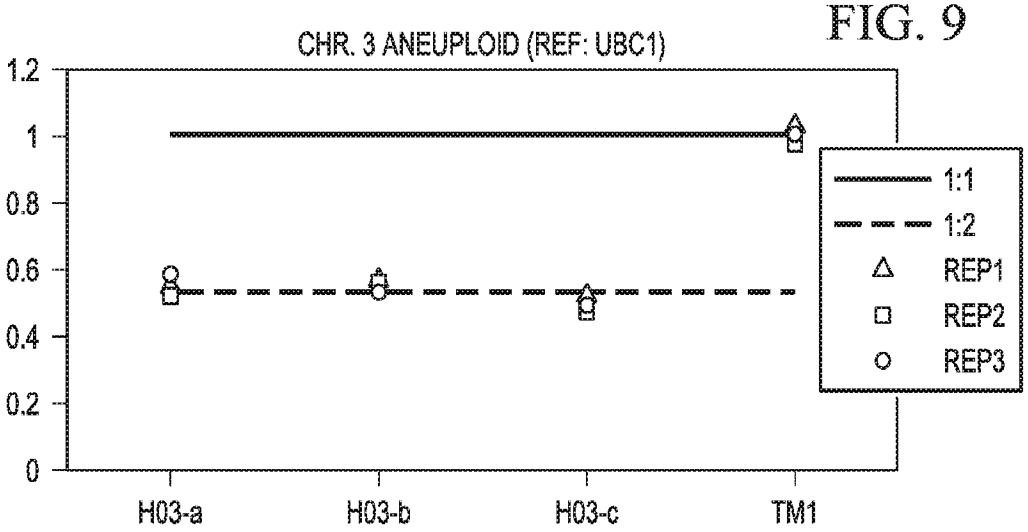


FIG. 10

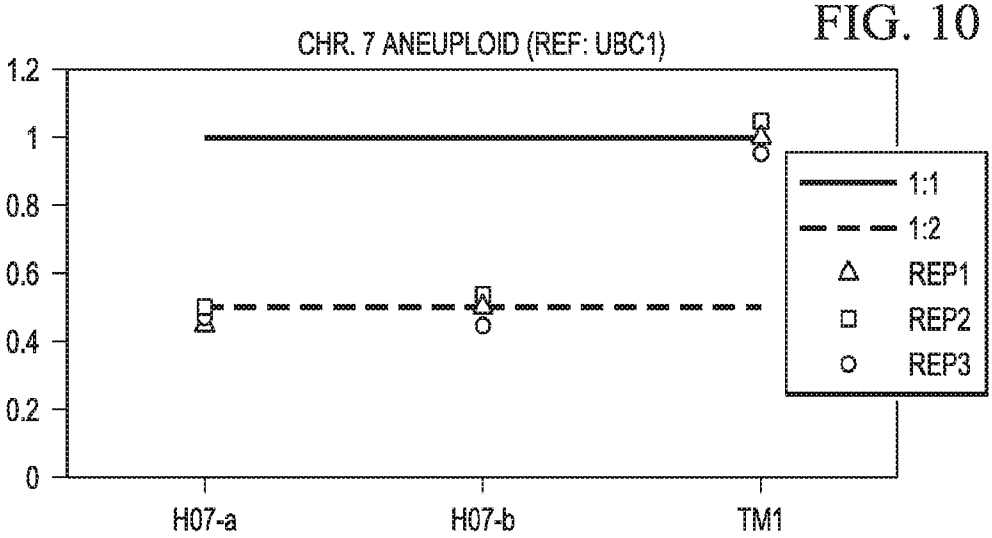


FIG. 11

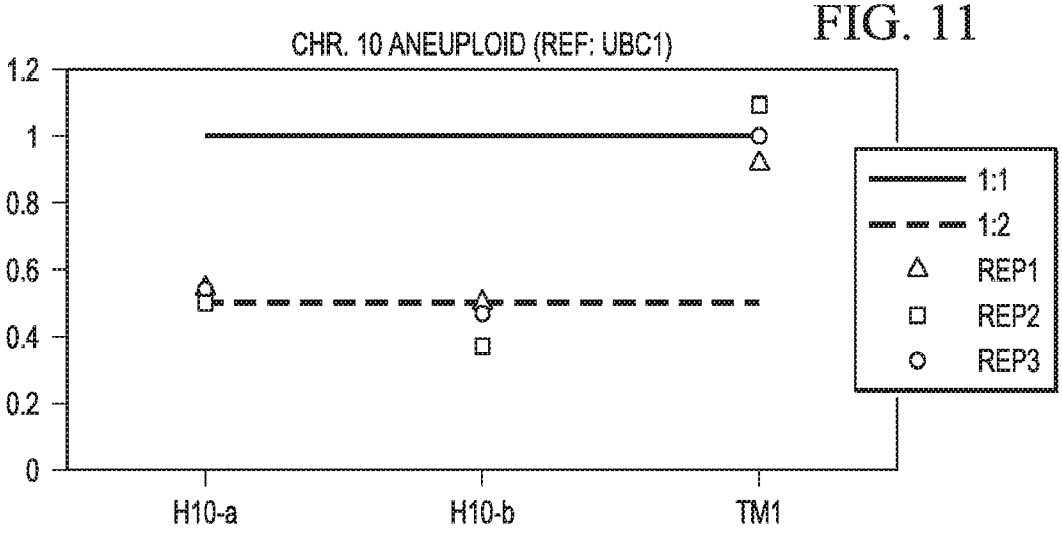


FIG. 12

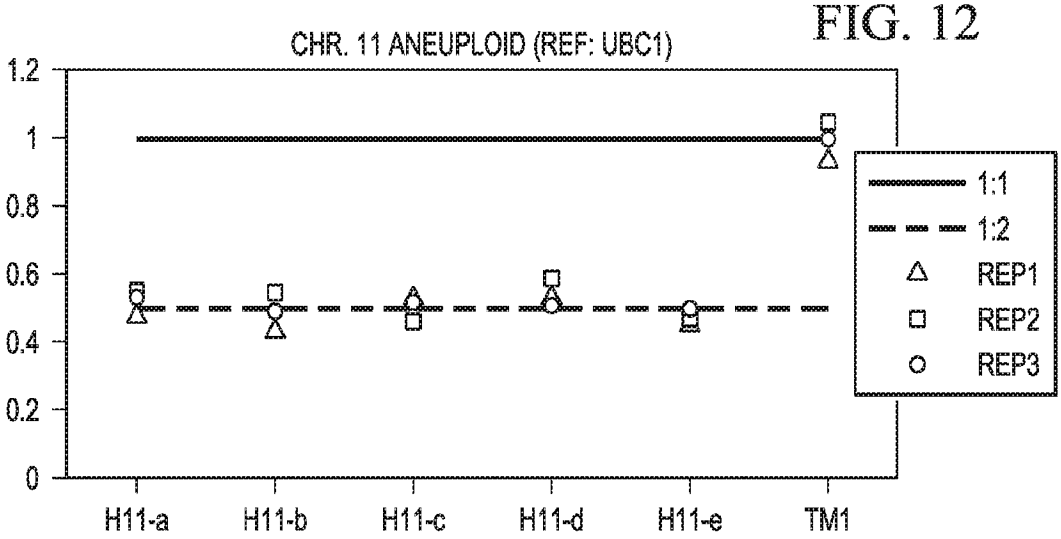


FIG. 13

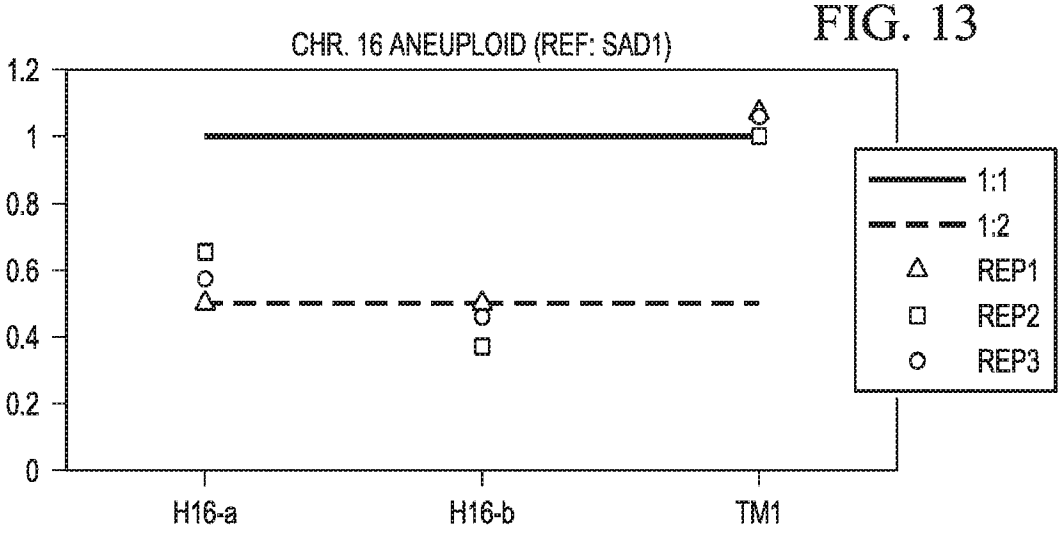


FIG. 14

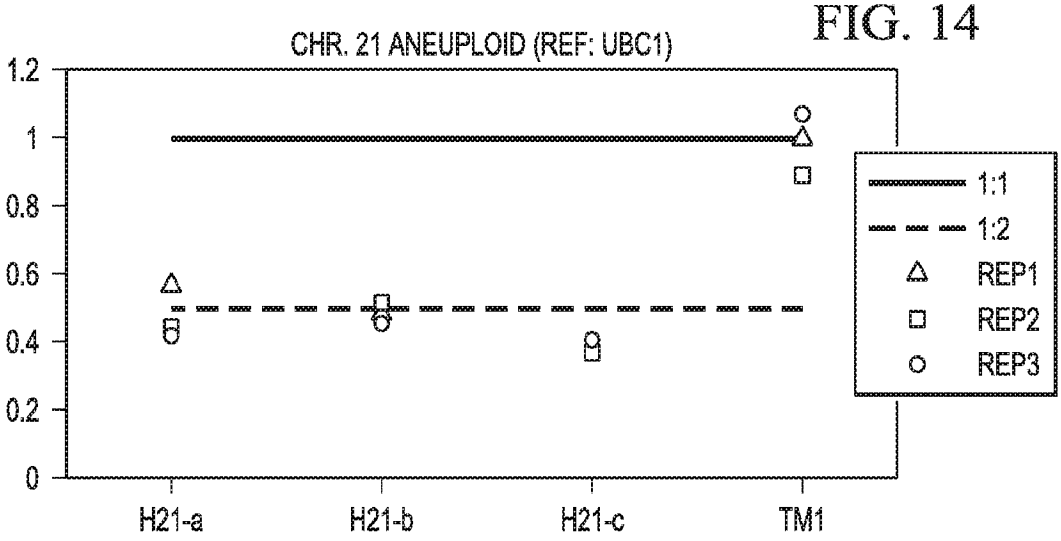


FIG. 15

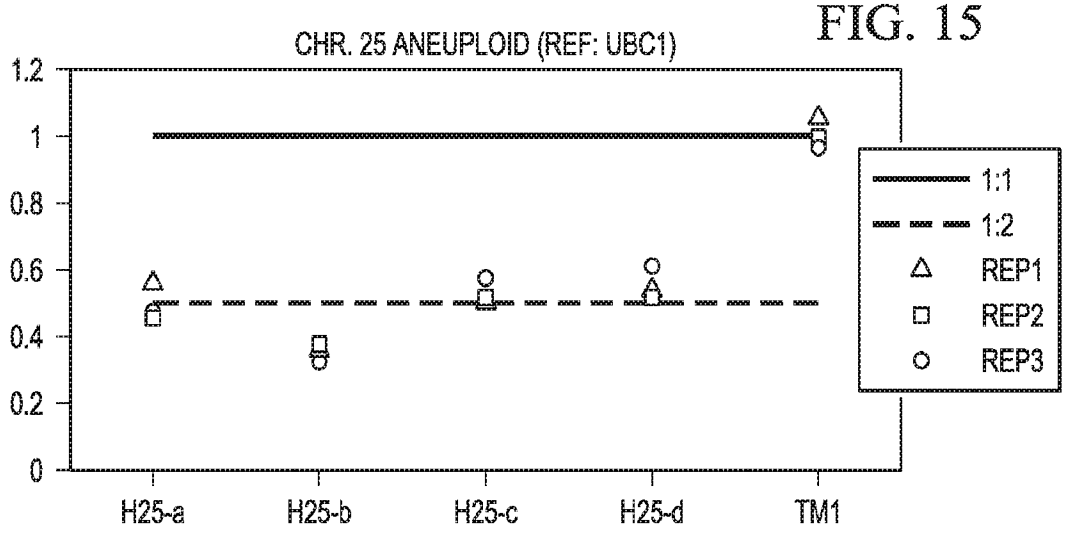


FIG. 16

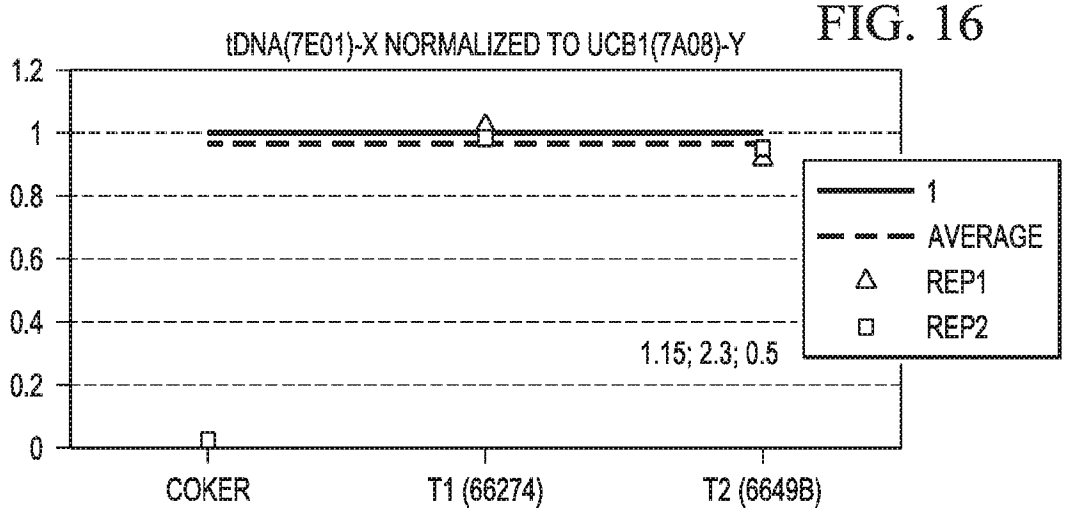


FIG. 17

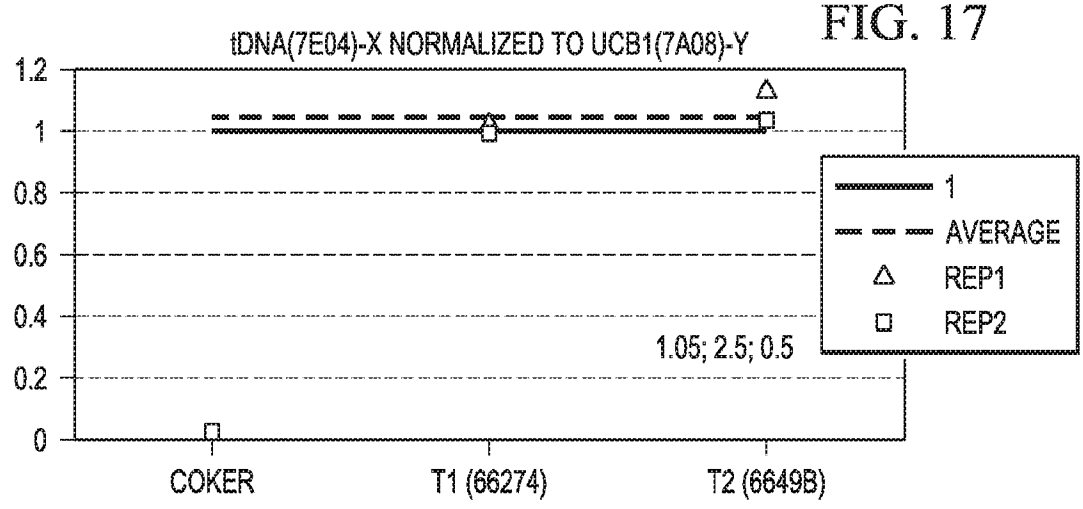


FIG. 18

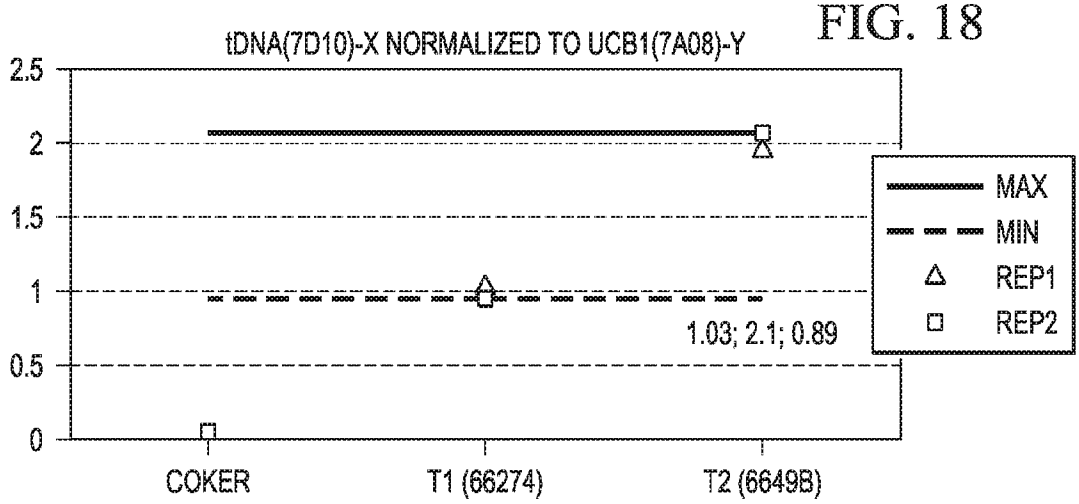

tDNA(7D10)-X NORMALIZED TO UCB1(7A08)-Y
2.5
2
1.5
1
0.5
0
1.03; 2.1; 0.89
MAX
MIN
REP1
REP2
COKER
T1 (66274)
T2 (6649B)

FIG. 19

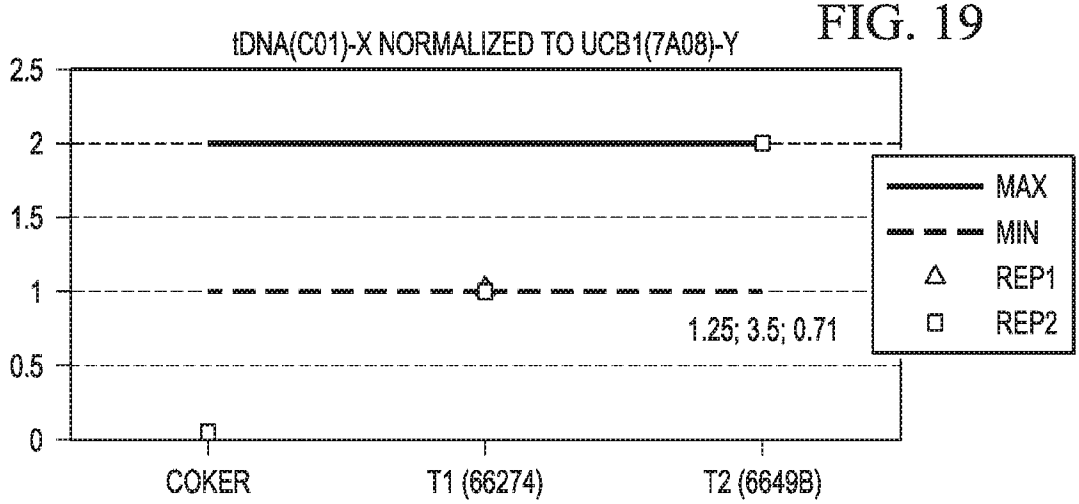

tDNA(C01)-X NORMALIZED TO UCB1(7A08)-Y
2.5
2
1.5
1
0.5
0
1.25; 3.5; 0.71
MAX
MIN
REP1
REP2
COKER
T1 (66274)
T2 (6649B)

FIG. 20

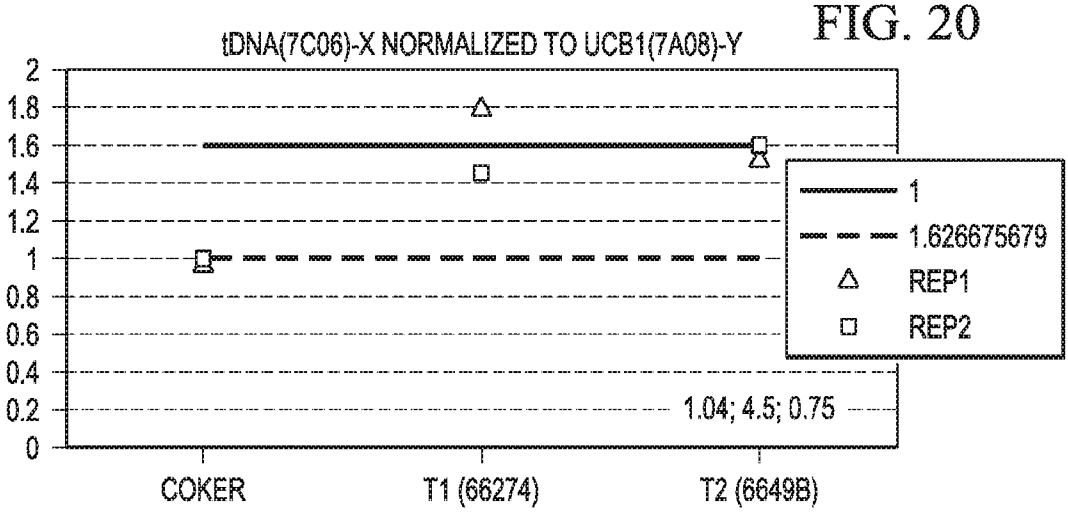
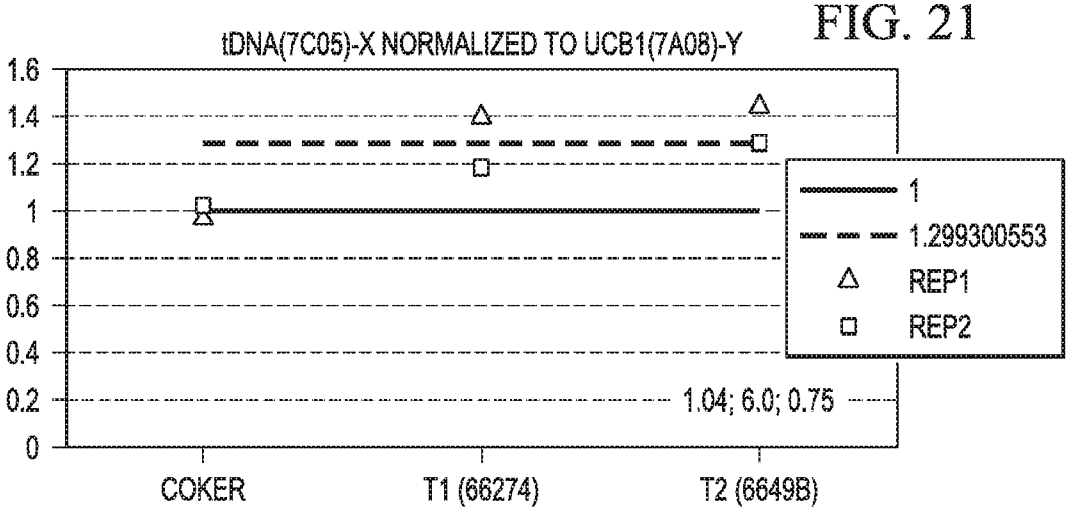

tDNA(7C06)-X NORMALIZED TO UCB1(7A08)-Y
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
1.626675679
REP1
REP2
1.04; 4.5; 0.75
COKER
T1 (66274)
T2 (6649B)

FIG. 21


tDNA(7C05)-X NORMALIZED TO UCB1(7A08)-Y
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
1.299300553
REP1
REP2
1.04; 6.0; 0.75
COKER
T1 (66274)
T2 (6649B)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/12298

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2017.01)
CPC - C12Q 1/682, 1/6876, 1/6895, 2600/13, 2600/124, 2600/16, 2600/156
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12Q 1/68 (2017.01)
CPC: C12Q 1/682, 1/6876, 1/6895, 2600/13, 2600/124, 2600/16, 2600/156

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C12Q 1/682, 1/6876, 1/6895, 2600/13, 2600/124, 2600/16, 2600/156 (text search)
USPC: 435/6.11; 536/23.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar Search terms: Copy number determination, variable copy number, defined copy number, gene marker of interest, fluorescent detection assay, Competitive Allele Specific PCR; Comparative Locus Amplification (CLA), fluorescent primer (e.g. FAM, HEX, VIC), reverse primer, quenching oli

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LGC Genomics. KASP genotyping chemistry User guide and manual [online]. 2013 [retrieved 2 March 2017]. Available on the internet: <URL: http://www.lgcgroup.com/LGCGroup/media/PDFs/Products/Genotyping/KASP-genotyping-chemistry-User-guide.pdf?ext=.pdf>. Especially pg 4 panels 1-4, pg 5 section 3 col 1 para 7, pg 6 section 3.9 para 3, pg 11 section 4.5.	1-20
Y	US 2011/0118145 A1 (AKMAEV et al.) 19 May 2011 (19.05.2011). Especially para [0046], [0092], [0095], claims 1, 2.	1-20
Y	Byers et al. Development and mapping of SNP assays in allotetraploid cotton. Theor Appl Genet May 2012 Vol 124 No 7 Pages 1201-1214. Especially pg 1202 col 1 para 3	5-7
Y	US 2007/0117108 A1 (ROBINSON) 24 May 2007 (24.05.2007). Especially [0036]	16-18

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2017	24 MAR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774