SYSTEMS AND METHODS FOR EVALUATING TUMOR FRACTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of United States Provisional Patent Application Serial No. 62/850,474, filed May 20, 2019, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Cancer cells accumulate mutations during cancer development and progression. These mutations may be the consequence of intrinsic malfunction of DNA repair, replication, or modification, or exposures to external mutagens. Certain mutations confer growth advantages on cancer cells and are positively selected in the microenvironment of the tissue in which the cancer arises. However, translating genomic studies to routine clinical practice still remains expensive, time intensive, and technically challenging.

[0003] Therefore, the need still exists for novel approaches, including genomic profiling, for analyzing samples associated with cancer.

SUMMARY

[0004] Methods and systems described herein allow for the evaluation of tumor fraction levels in a sample, biopsy or subject. Typically, tumor fraction is expressed or measured as the level or proportion of tumor-derived DNA in a sample relative to a reference, e.g., non-tumor DNA or all DNA, in the sample. In the methods described herein, a value for a certainty metric for the sample is obtained and that value can be evaluated in terms of a reference, e.g., by being compared with a reference. A certainty metric can itself be a function of a target variable that reflects the level of an allele at a subgenomic interval. Target variables may include variables that are a function of allele fraction, as well as variables that are a function of reads of subgenomic intervals.

[0005] In some examples, a value for the target variable is acquired, e.g., directly acquired, from the sample. Typically, the reference against which the certainty metric for the sample is compared is a certainty metric value (or a plurality of certainty metric values) that is associated with, e.g., correlated to, a level of tumor fraction. Certainty metric values that are incorporated

into the reference can be based, e.g., on entities or relationships within the sample (e.g., 0.5 for an allele at a heterologous subgenomic interval) or external to the sample (e.g., a standard curve made from one or more other subjects).

[0006] In some examples, the target variable may be an allele fraction at one or more subgenomic intervals. Other examples of target variable include variables like log2ratio, which is a function of the number of reads at one or more subgenomic intervals. Typically, a plurality of subgenomic intervals (e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, or more subgenomic intervals) are analyzed to determine tumor faction. The plurality of subgenomic intervals may be present on the same chromosome or on different chromosomes (e.g., distributed among 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or more chromosomes). In an example, at least a portion of the plurality of subgenomic intervals are heterozygous (in terms of the alleles at the subgenomic interval).

[0007] In an example, a certainty metric for a sample from a subject is compared with a curve that relates certainty metric to tumor fraction, and a value for sample tumor fraction is obtained.

[0008] In an example, the certainty metric is a function of a target variable, e.g., allele fraction. By way of example, the certainty metric can be related to the degree by which the observed allele fraction deviates from a reference, e.g., an expected allele fraction or log2ratio, and compared with a reference associated with a level of tumor fraction. In other examples, the certainty metric may measure the relative certainty of the target variable, e.g., an entropy metric described herein.

[0009] Thus, methods described herein include methods of evaluating, e.g., estimating, the tumor fraction of a sample. Such methods include, for example:

obtaining a value for a target variable of a sample;

obtaining a value for a reference, e.g., certainty metric as a function of the target variable; and

comparing the sample value with the reference value to obtain a value for the tumor fraction of the sample.

[0010] In some examples, a method of determining a tumor fraction of a sample from a subject comprises acquiring a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample; determining a certainty metric

indicative of a dispersion of the plurality of values; accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample. In one aspect, the present disclosure refers to a method of determining a tumor fraction of a sample from a subject, comprising: acquiring a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample; determining a certainty metric indicative of a dispersion of the plurality of values; accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

[0011] In some examples, each value within the plurality of values is an allele fraction. In some examples, each value within the plurality of values comprises a ratio of the difference in abundance between a maternal allele and a paternal allele relative to abundance of the maternal allele or the paternal allele at the corresponding locus. In some examples, the certainty metric is indicative of a deviation of each of the plurality of values from an expected value. In some examples, the expected value is a locus-specific expected value.

[0012] In some examples, the certainty metric is a root mean squared deviation from the expected value. In some examples, the expected value is an expected allele frequency for a non-tumorous. In some examples, each value within the plurality of values is and allele fraction, and the expected value is 0.5.

[0013] In some examples, each value within the plurality of values is a ratio of the difference in abundance between a maternal allele and a paternal allele, relative to abundance of the maternal allele or the paternal allele at the corresponding locus, and the expected value comprises the expected ratio of the difference in abundance between a maternal allele and a paternal allele relative, to abundance of the maternal allele or the paternal allele, wherein the expected value is the expected ratio for a non-tumorous sample. In some examples, the expected value is 0.

[0014] In some examples, the plurality of values comprises a plurality of allele coverages.

[0015] In some examples, the method further comprising determining a probability distribution function for the plurality of values; wherein the certainty metric is determined using the probability distribution function. In some examples, the certainty metric is an entropy of the probability distribution function.

[0016] In some examples, the corresponding loci comprise one or more loci having a different maternal allele and paternal allele. In some examples, the corresponding loci consist of loci having a different maternal allele and paternal allele. In some examples, the corresponding loci comprise one or more loci having the same maternal allele and paternal allele.

[0017] In some examples, a method of determining a tumor fraction of a sample from a subject, comprises: acquiring a plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval; determining a certainty metric indicative of a dispersion of the plurality of values; accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample. In one aspect, the present disclosure refers to a method of determining a tumor fraction of a sample from a subject, comprising: acquiring a plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval; determining a certainty metric indicative of a dispersion of the plurality of values; accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

[0018] In some examples, each value within the plurality of values comprises a ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample.

[0019] In some examples, each value within the plurality of values comprises a log ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample. In some examples, the log ratio is a log2 ratio.

[0020] In some examples, each value within the plurality of values comprises a ratio of the difference in an allele coverage the locus in the tumor sample and same locus in the non-tumor sample, relative to an allele coverage of the same locus in the non-tumor sample.

[0021] In some examples, the certainty metric is indicative of a deviation of each value within the plurality of values from an expected value across the corresponding loci, wherein the expected value is the value that would be expected if the tumor sample were a non-tumor sample.

[0022] In some examples, each value comprises a ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample, and the expected value is 1; each value comprises a log ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample, and the expected value is 0; or each value comprises a ratio of the difference in an allele coverage the locus in the tumor sample and same locus in the non-tumor sample, relative to an allele coverage of the same locus in the non-tumor sample, and the expected value is 0.

[0023] In some examples, the certainty metric is a root mean squared deviation from the expected value.

[0024] In some examples, the method further comprising determining a probability distribution function for the plurality of values; wherein the certainty metric is determined using the probability distribution function. In some examples, the certainty metric is an entropy of the probability distribution function.

[0025] In some examples, the allele coverage comprises an allele coverage of a maternal allele and a paternal allele.

[0026] In some examples, the allele coverage consists of an allele coverage of a maternal allele and a paternal allele.

[0027] In some examples of the above methods, the plurality of loci comprises at least one nucleotide associated with a single nucleotide polymorphism (SNP). In some examples, the plurality of loci comprises two or more nucleotides each associated with a single nucleotide polymorphism (SNP). In some examples, the SNP is associated with a cancer.

[0028] In some examples of the above methods, at least a portion of the plurality of loci is associated with a copy number variation (CNV). In some examples, the CNV is associated with a cancer.

[0029] In some examples of the above methods, the method further comprises sequencing the sample, to determine an allele abundance or coverage at each locus.

[0030] In some examples of the above methods, the method further comprises performing array hybridization on the sample to determine an allele abundance or coverage at each locus.

[0031] In some examples of the above methods, the method further comprises accessing a training dataset comprising a plurality of relationships between a plurality of training certainty metrics and associated training tumor fractions; and applying a machine learning process to the training dataset to determine the predetermined relationship between the training certainty metrics and the training tumor fractions.

[0032] In some examples of the above methods, the method further comprises generating a report comprising information identifying the subject and the determined tumor fraction. In some examples, the method further comprises providing the report to the subject or a healthcare provider. In some examples, the method further comprises formatting the report for an electronic health record.

[0033] In some examples, a method of treating a tumor in a subject comprises, responsive to a determined tumor fraction, administering an effective amount of a tumor therapy to the subject,

wherein the tumor fraction is determined according to any one of the methods described above. In some examples, the method comprises determining, based on the determined tumor fraction, the presence of the tumor in the patient. In some examples, the tumor therapy comprises chemotherapy, radiation therapy, or surgery.

[0034] In some examples, a method of monitoring tumor progression or recurrence in a subject comprises (a) determining a first tumor fraction of a first sample obtained from the subject at a first time point according to any one of the methods described above; (b) determining a second tumor fraction of a second sample obtained from the subject at a second time point; and (c) comparing the first tumor fraction to the second tumor fraction, thereby monitoring the tumor progression. In one aspect, the present disclosure refers to a method of monitoring tumor progression or recurrence in a subject, comprising: (a) determining a first tumor fraction of a first sample obtained from the subject at a first time point according to the method as disclosed herein; (b) determining a second tumor fraction of a second sample obtained from the subject at a second time point; and (c) comparing the first tumor fraction to the second tumor fraction, thereby monitoring the tumor progression.

[0035] In some examples of the method of monitoring tumor progression or recurrence, determining the second tumor fraction comprises acquiring a second plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the second tumor sample, wherein the subgenomic interval in the second sample is the same or different than the subgenomic interval in the first sample; determining a second certainty metric indicative of a dispersion of the second plurality of values; accessing the predetermined relationship between one or more stored certainty metrics and one or more stored tumor fractions; and determining, from the second certainty metric and the predetermined relationship, the second tumor fraction of the second sample.

[0036] In some examples of the method of monitoring tumor progression or recurrence, determining the second tumor fraction comprises acquiring a second plurality of values, each value indicative of a difference between an allele coverage of a locus in the second tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval in the sample, wherein the subgenomic interval used to determine the second tumor fraction is the same or different than the subgenomic interval used to determine the first tumor fraction; determining a second certainty metric indicative of a dispersion of the second plurality of values; accessing the predetermined relationship between one or more stored certainty metrics and one or more stored tumor fractions; and determining, from the second certainty metric and the predetermined relationship, the second tumor fraction of the second tumor sample.

[0037] In some examples of the method of monitoring tumor progression or recurrence, the method further comprises adjusting a tumor therapy in response to the tumor progression. In some examples, the method comprises adjusting a dosage of the tumor therapy or selecting a different tumor therapy in response to the tumor progression. In some examples, the method comprises administering the adjusted tumor therapy to the subject.

[0038] In some examples of the method of monitoring tumor progression or recurrence the method comprises the first time point is before the subject has been administered a tumor therapy, and wherein the second time point is after the subject has been administered the tumor therapy.

[0039] In some examples of any of the methods described above, the subject has a cancer, is at risk of having a cancer, or is suspected of having a cancer. In some examples, the cancer is a solid tumor. In some examples, the cancer is a hematological cancer.

[0040] In some examples of any of the methods described above,the sample is a liquid sample.

[0041] In some examples of any of the methods described above,the sample is a solid sample.

[0042] In some examples of any of the methods described above,the sample comprises cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA).

[0043] In some examples of any of the methods described above, the one or more stored certainty metrics comprises a plurality of stored certainty metrics, and the one or more stored tumor fractions comprises plurality of stored tumor fractions.

[0044] Also described herein is a computer system comprising: a processor; and a memory communicatively coupled to the processor, configured to store: a predetermined relationship between a one or more stored certainty metric and one or more associated stored tumor fraction; and instructions that, when executed by the processor cause the processor to: (a)(i) acquire a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample, or (ii) acquire plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval; (b) determine a certainty metric indicative of a dispersion for the plurality of values; (c) access the

stored predetermined relationship; and (d) determine, from the certainty metric and the predetermined relationship, the tumor fraction of the sample. In one aspect, the present disclosure refers to a computer system comprising: a processor; and a memory communicatively coupled to the processor, configured to store: a predetermined relationship between a one or more stored certainty metric and one or more associated stored tumor fraction; and instructions that, when executed by the processor cause the processor to: (a)(i) acquire a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample, or (ii) acquire plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval; (b) determine a certainty metric indicative of a dispersion for the plurality of values; (c) access the stored predetermined relationship; and (d) determine, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

[0045] In some examples, of the computer system, the memory further comprises instructions that, when executed by the processor, cause the processor to: access a training dataset comprising a plurality of relationships between a plurality of training certainty metrics and associated training tumor fractions; and apply a machine learning process to the training dataset to determine the predetermined relationship between the training certainty metrics and the training tumor fractions.

[0046] In some examples of the computer system, the instructions, when executed by the processor, cause the processor to perform any one of the methods described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] Various aspects of at least one example are discussed below with reference to the accompanying figures, which are not intended to be drawn to scale. The figures are included to provide an illustration and a further understanding of the various aspects and examples, and are incorporated in and constitute a part of this specification, but are not intended as a definition of the limits of a particular example. The drawings, together with the remainder of the specification, serve to explain principles and operations of the described and claimed aspects and examples. In the figures, each identical or nearly identical component that is illustrated in various figures is represented by a like numeral. For purposes of clarity, not every component may be labeled in every figure.

[0048] FIG. 1 depicts a process according to one embodiment. The disclosed process may be used to estimate a tumor fraction from a sample.

[0049] FIG. 2 shows an example computer system with which various aspects of the present disclosure may be practiced.

[0050] FIG. 3 shows an example storage system capable of implementing various aspects of the present disclosure.

[0051] FIG. 4 shows an exemplary relationship between entropy of a probability distribution function for SNP allele fractions in a sample with an associated tumor fraction (as represented by maximum somatic allele frequency), as determined using several serially diluted cancer samples.

CLAIMS

1. A method of determining a tumor fraction of a sample from a subject, comprising:

acquiring a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample;

determining a certainty metric indicative of a dispersion of the plurality of values;

accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and

determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

2. The method of claim 1, wherein:

each value within the plurality of values is an allele fraction; or

each value within the plurality of values comprises a ratio of the difference in abundance between a maternal allele and a paternal allele relative to abundance of the maternal allele or the paternal allele at the corresponding locus.

3. The method of claim 1 or 2, wherein the certainty metric is indicative of a deviation of each of the plurality of values from an expected value:

optionally wherein:

the expected value is a locus-specific expected value; or

the certainty metric is a root mean squared deviation from the expected value; or

the expected value is an expected allele frequency for a non-tumorous sample; or

each value within the plurality of values is and allele fraction, and the expected value is 0.5; or

each value within the plurality of values is a ratio of the difference in abundance between a maternal allele and a paternal allele, relative to abundance of the maternal allele or the paternal allele at the corresponding locus, and the expected value comprises the expected ratio of the difference in abundance between a maternal allele

and a paternal allele relative, to abundance of the maternal allele or the paternal allele, wherein the expected value is the expected ratio for a non-tumorous sample; or

the plurality of values comprises a plurality of allele coverages.

4. The method of any one of claims 1-3, the method further comprising determining a probability distribution function for the plurality of values; wherein the certainty metric is determined using the probability distribution function;

optionally wherein the certainty metric is an entropy of the probability distribution function.

5. The method of any one of claims 1-4, wherein:

the corresponding loci comprise one or more loci having a different maternal allele and paternal allele; or

the corresponding loci consist of loci having a different maternal allele and paternal allele; or

the corresponding loci comprise one or more loci having the same maternal allele and paternal allele.

6. A method of determining a tumor fraction of a sample from a subject, comprising:

acquiring a plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval;

determining a certainty metric indicative of a dispersion of the plurality of values;

accessing a predetermined relationship between one or more stored certainty metric and one or more stored tumor fraction; and

determining, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

7. The method of claim 6, wherein:

each value within the plurality of values comprises a ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample; or

each value within the plurality of values comprises a log ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample; or

each value within the plurality of values comprises a ratio of the difference in an allele coverage the locus in the tumor sample and same locus in the non-tumor sample, relative to an allele coverage of the same locus in the non-tumor sample; or

the certainty metric is indicative of a deviation of each value within the plurality of values from an expected value across the corresponding loci, wherein the expected value is the value that would be expected if the tumor sample were a non-tumor sample, optionally wherein:

(i) each value comprises a ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample, and the expected value is 1; or

(ii) each value comprises a log ratio of an allele coverage of a locus in the tumor sample compared to an allele coverage of the same locus in the non-tumor sample, and the expected value is 0; or

(iii) each value comprises a ratio of the difference in an allele coverage the locus in the tumor sample and same locus in the non-tumor sample, relative to an allele coverage of the same locus in the non-tumor sample, and the expected value is 0.

8. The method of claim 7, the method further comprising determining a probability distribution function for the plurality of values; wherein the certainty metric is determined using the probability distribution function;

optionally wherein the certainty metric is an entropy of the probability distribution function.

9. The method of claim 7 or 8, wherein:

the allele coverage comprises an allele coverage of a maternal allele and a paternal allele; or

the allele coverage consists of an allele coverage of a maternal allele and a paternal allele; or

the allele coverage consists of an allele coverage of a maternal allele and a paternal allele.

10. The method of any one of claims 1-9, wherein:

the plurality of loci comprises at least one nucleotide associated with a single nucleotide polymorphism (SNP); optionally wherein (i) the plurality of loci comprises two or more nucleotides each associated with a single nucleotide polymorphism (SNP), and/or (ii) the SNP is associated with a cancer; or

at least a portion of the plurality of loci is associated with a copy number variation (CNV), optionally wherein the CNV is associated with a cancer.

11. The method of any of claims 1-10, further comprising:

sequencing the sample, to determine an allele abundance or coverage at each locus; or

performing array hybridization on the sample to determine an allele abundance or coverage at each locus.

12. The method of any of claims 1-11, further comprising:

accessing a training dataset comprising a plurality of relationships between a plurality of training certainty metrics and associated training tumor fractions; and

applying a machine learning process to the training dataset to determine the predetermined relationship between the training certainty metrics and the training tumor fractions.

13. The method of any one of claims 1-12, comprising generating a report comprising information identifying the subject and the determined tumor fraction; and

optionally comprising providing the report to the subject or a healthcare provider, and/or formatting the report for an electronic health record.

14. A method of monitoring tumor progression or recurrence in a subject, comprising:

(a) determining a first tumor fraction of a first sample obtained from the subject at a first time point according to the method of any one of claims 1-13;

(b) determining a second tumor fraction of a second sample obtained from the subject at a second time point; and

(c) comparing the first tumor fraction to the second tumor fraction, thereby monitoring the tumor progression.

15. The method of claim 14, wherein:

(a) determining the second tumor fraction comprises:

acquiring a second plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the second tumor sample, wherein the subgenomic interval in the second sample is the same or different than the subgenomic interval in the first sample;

determining a second certainty metric indicative of a dispersion of the second plurality of values;

accessing the predetermined relationship between one or more stored certainty metrics and one or more stored tumor fractions; and

determining, from the second certainty metric and the predetermined relationship, the second tumor fraction of the second sample; or

(b) determining the second tumor fraction comprises:

acquiring a second plurality of values, each value indicative of a difference between an allele coverage of a locus in the second tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval in the sample, wherein the subgenomic interval used to determine the second tumor fraction is the same or different than the subgenomic interval used to determine the first tumor fraction;

determining a second certainty metric indicative of a dispersion of the second plurality of values;

accessing the predetermined relationship between one or more stored certainty metrics and one or more stored tumor fractions; and

determining, from the second certainty metric and the predetermined relationship, the second tumor fraction of the second tumor sample.

16. The method of claim 14 or 15, wherein the first time point is before the subject has been administered a tumor therapy, and wherein the second time point is after the subject has been administered the tumor therapy.

17. The method of any one of claims 1-16, wherein:

the subject has a cancer, is at risk of having a cancer, or is suspected of having a cancer; or

the sample is a liquid sample; or

the sample is a solid sample; or

the sample comprises cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA); or the one or more stored certainty metrics comprises a plurality of stored certainty metrics, and the one or more stored tumor fractions comprises plurality of stored tumor fractions.

18. A computer system comprising:

a processor; and

a memory communicatively coupled to the processor, configured to store:

a predetermined relationship between a one or more stored certainty metric and one or more associated stored tumor fraction; and

instructions that, when executed by the processor cause the processor to:

(a)(i) acquire a plurality of values, each value indicative of an allele fraction at a corresponding locus within a subgenomic interval in the sample, or (ii) acquire plurality of values, each value indicative of a difference between an allele coverage of a locus in a tumor sample and an allele coverage of the same locus in a non-tumor sample at a plurality of loci within a subgenomic interval;

(b) determine a certainty metric indicative of a dispersion for the plurality of values;

(c) access the stored predetermined relationship; and

(d) determine, from the certainty metric and the predetermined relationship, the tumor fraction of the sample.

19. The computer system of claim 18, wherein the memory further comprises instructions that, when executed by the processor, cause the processor to:

access a training dataset comprising a plurality of relationships between a plurality of training certainty metrics and associated training tumor fractions; and

apply a machine learning process to the training dataset to determine the predetermined relationship between the training certainty metrics and the training tumor fractions.

20. The computer system of claim 18 or 19, wherein the instructions, when executed by the processor, cause the processor to perform the method of any one of claims 1-13.