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(54) Title: METHOD AND MATERIALS FOR QUATERNARY AMINE CATALYZED BISULFITE CONVERSION OF CYTOSINE TO URACIL

(57) Abstract: The invention provides methods and materials for the conversion of cytosine to uracil. A nucleic acid, such as gDNA, is reacted with bisulfate, such as magnesium bisulfite, in the presence of a quaternary amine catalyst. Examples of suitable quaternary amine catalysts include but are not limited to quaternary ammonium compounds, quaternary alkyl ammonium salts, quaternary alkyl ammonium halides, quaternary methyl ammonium bromide, quaternary ammonium chloride, tetraethyl ammonium hydroxide, tetraethylammonium chloride, tetrabutyl ammonium chloride, tetrabutyl ammonium bromide. The invention also contemplates kits of premeasured ingredients for carrying out the methods of the invention either on an individual sample or on a plurality of samples.

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**METHOD AND MATERIALS FOR QUATERNARY AMINE
CATALYZED BISULFITE CONVERSION OF CYTOSINE TO URACIL**

[001] This application claims benefit of priority to US Provisional Application Serial Nos. 60/499,106 filed August 29, 2003 and 60/523,054 filed November 17, 2003, each of which is hereby incorporated by reference.

FIELD

[002] The invention relates generally to methods and materials for the conversion of cytosine to uracil.

BACKGROUND

[003] Assessment of methylation of DNA is useful in many research, diagnostic, medical, forensic, and industrial fields. Key to this assessment is converting cytosine, but not methylcytosine, to uracil, but not thymine. One basic method for such conversion, employing sodium bisulfite, is well known. Over the years, the method has been improved in attempts to overcome disadvantages that include tedious procedures, lengthy reaction times, and DNA degradation. The most commonly used protocol is taught by J. Herman, *Proc. Natl. Acad. Sci.* **93**, 9821-26 (1996), incorporated herein by reference in its entirety. This method involves denaturation, reaction with sodium bisulfite in the presence of hydroquinone, and subsequent completion of the modification by treatment with NaOH. Despite the attempts to improve the protocol, current procedures require pre-denaturation of the genomic DNA (gDNA) to single stranded DNA (ssDNA), preparation of fresh solutions of sodium bisulfite (NaHSO₃), typically about 3M, and inclusion of an anti-oxidant (e.g., hydroquinone). The protocol also requires long reaction times and tedious clean-up procedures.

[004] In addition, the currently employed sodium bisulfite protocols are plagued by reports of incomplete conversion, irreproducible results, and other problems. In some cases, the reaction can result in significant DNA degradation (reportedly as high as 96%), making it difficult to obtain enough sample for further analysis. See. S.J. Clark et al. *Nucleic Acid Research* 2001, **29** no. 13, e65. Given the importance of assessment of DNA methylation, it can be seen that there is a need for improved methodologies for conversion of cytosine to uracil.

[005] It has been discovered that bisulfite methods that employ magnesium bisulfite, polyamine compounds, and/or quaternary amine compounds provide useful alternatives to sodium bisulfite conversion reactions. These discoveries are the subjects of co-owned applications entitled "Method And Materials For Polyamine Catalyzed Bisulfite Conversion Of Cytosine To Uracil" (U.S. application Ser. No. 60/499,113 filed August 29, 2003, and also application Ser. No. 60/499,113 (docket no. 5065P2) having the same title and filed November 17, 2003), "Method And Materials For Quaternary Amine Catalyzed Bisulfite Conversion Of Cytosine To Uracil" (U.S. application Ser. No. 60/499,106 filed August 29, 2003, and "Method and Materials for Bisulfite Conversion of Cytosine to Uracil" (U.S. application Ser. No. 60/499,082 filed August 29, 2003, and also application Ser. No. 60/523,056 having the same title and filed November 17, 2003), all of which are hereby incorporated by reference in their entirety. Improvements in clean-up procedures associated with conversion of cytosine to uracil are also the subject of co-owned applications entitled "Improved Bisulfite Method" (U.S. application Ser. No. 60/498,996 filed August 29, 2003, and also application Ser. No. 60/520,941 (5109P2) having the same title and filed November 17, 2003) all of which are hereby incorporated by reference in their entirety.

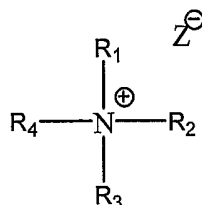
SUMMARY

[006] In certain embodiments of the invention, the invention comprises methods of specifically converting cytosine to uracil by using a catalyzed bisulfite reaction.

[007] In some embodiments, the present invention provides methods for the conversion of cytosine to uracil in a nucleic acid comprising the steps of:

[008] reacting a nucleic acid comprising at least one cytosine nucleobase with bisulfite ion in the presence of a quaternary amine catalyst.

[009] In some embodiments, the quaternary amine comprises a compound having Formula I:



Formula I

[010] or a derivative thereof, wherein:

[011] R_1 , R_2 , R_3 and R_4 are each independently alkyl, preferably C_1 - C_4 alkyl; and

[012] Z^{\ominus} is selected from the halides and OH^- .

[013] In some embodiments, R_1 , R_2 , R_3 and R_4 are identical.

[014] In some embodiments of the invention, the quaternary amine catalyst comprises a quaternary ammonium compound, or a derivative thereof. In further embodiments, the quaternary amine catalyst comprises a quaternary alkyl ammonium salt. In yet further embodiments, the quaternary amine catalyst comprises a quaternary alkyl ammonium halide, for example a quaternary ammonium chloride or a quaternary ammonium bromide. In some embodiments, the quaternary amine catalyst comprises at least one of quaternary methyl ammonium bromide, tetraethyl ammonium hydroxide, tetraethylammonium chloride, tetrabutyl ammonium chloride and tetrabutyl ammonium bromide.

[015] In some embodiments, the reaction of the nucleic acid and bisulfite ion is performed in a solution containing bisulfite ion, such as magnesium bisulfite, in a

concentration of from about 0.5M to about 2.5M. In further embodiments, the solution contains bisulfite ion, such as magnesium bisulfite, in a concentration of from about 1M to about 2M.

[016] In some embodiments, the magnesium bisulfite is present at a concentration of at least about 1M.

[017] Also provided are methods for the conversion of cytosine to uracil comprising the steps of reacting a DNA sample in solution with a bisulfite salt and quaternary amine catalyst as described above, wherein the concentration of the bisulfite salt is from about 0.5M to about 2M. In further embodiments, the concentration of the bisulfite salt is about 1.3M.

[018] In some embodiments of the methods of the invention, the reaction is performed at a temperature from about 40 to about 60 degrees, such as about 50 degrees, for about 4 to about 15 hours. In further embodiments of the methods of the invention, the nucleic acid is gDNA.

[019] Also provided in accordance with the present invention are kits for use in conversion of cytosine to uracil comprising magnesium bisulfite; and a quaternary amine catalyst as described above. In some embodiments of such kits, the magnesium bisulfite is provided as an approximately 2M magnesium bisulfite solution. In further embodiments of the kits of the invention, the quaternary amine catalyst comprises tetraethyl ammonium hydroxide. . In some embodiments, the kits further comprise reagents for sequencing and/or amplification (e.g., by PCR), for example a polymerase and one or more primers. In some embodiments, the kits contain premeasured materials useful in various embodiments of the methods of the invention.

[020] In some embodiments, the methylation status of one or more cytosines in the target nucleic acid(s) can be determined by any suitable method. Typically, methylation status can be determined by measuring the presence or relative amount of uracil at a nucleotide position that was previously non-methylated cytosine, and was converted to uracil

by the bisulfite treatment. If desired, the presence or relative amount of residual cytosine at the same nucleotide position (indicating the presence of methylcytosine) can be measured for comparison with the amount of uracil, to determine the degree of methylation at the particular nucleotide position. Appropriate control experiments can also be performed to correct for incomplete transformation of cytosine to uracil, if desired.

[021] The presence or amount of uracil and/or methylcytosine at a particular nucleotide position can be measured by any suitable method, such as DNA sequencing (e.g., by the Sanger method or Maxam-Gilbert method or subsequent embodiments thereof (e.g., using dye-labeled terminators or dye-labeled primers, such as discussed in WO 02/30944 and by Ansorge et al. *DNA Sequencing Strategies – Automated and Advanced Approaches*, John Wiley & Sons, New York, 1997)), PCR (e.g., primer-specific PCR), oligonucleotide ligation assay (OLA) or other ligation-dependent techniques (e.g., see U.S. Patent No. 6,511,810 and references cited therein), single base extension (over the potential methylation site), mass spectrometry, real time PCR (e.g., using labeled probes that are complementary to C and or U), microarrays comprising sequence specific probes, etc. Various exemplary techniques are also described by Kirk et al., *Nucl. Acids Res.*, 30:3295-3311 (2002).

DETAILED DESCRIPTION

[022] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. In this application, the use of “or” means “and/or” unless stated otherwise. Furthermore, the use of the term “including”, as well as other forms, such as “includes” and “included”, is not limiting. Also, terms such as “element” or

“component” encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise.

[023] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose.

DEFINITIONS

[024] As used herein, the term “alkyl” is intended to mean saturated hydrocarbon species, including without limitation straight, branched chain and cyclic hydrocarbons, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, t-butyl, n-pentyl, sec-pentyl, t-pentyl, neopentyl, cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl groups.

[025] As used herein, the term "EXO/SAP" denotes a mixture of exonuclease I (EXO) and shrimp alkaline phosphatase (SAP).

[026] As used herein, the term “gDNA” refers to genomic DNA.

[027] Bisulfite ion has its accustomed meaning of HSO_3^- . Typically, bisulfite is used as an aqueous solution of a bisulfite salt, for example magnesium bisulfite, which has the formula $\text{Mg}(\text{HSO}_3)_2$, and sodium bisulfite, which has the formula NaHSO_3 .

[028] The term “PCR” is intended to denote polymerase chain reaction, as is well known in the art. The term “MSP” denotes methylation specific PCR, such as described by J. Herman, *Proc. Natl. Acad. Sci.* **93**, 9821-26 (1996), and modified as discussed herein.

[029] As used herein, the term “nucleic acid” includes, for example, nucleobase-containing polymeric compounds, including naturally occurring and non-naturally occurring forms thereof, for example and without limitation, genomic DNA, cDNA, hnRNA, mRNA,

rRNA, tRNA, fragmented nucleic acids, nucleic acids obtained from subcellular organelles such as mitochondria or chloroplasts, and nucleic acids obtained from microorganisms, or DNA or RNA viruses that may be present on or in a biological sample.

[030] The term “quaternary amine compound” or “quaternary amine” is intended to include, without limitation, compounds containing a tetra-substituted nitrogen atom, and the salts and hydroxides of such compounds. Examples of quaternary amine compounds include, without limitation, quaternary alkyl ammonium compounds, for example quaternary alkyl ammonium halides. Thus, quaternary amine compounds include quaternary alkyl ammonium chlorides such as quaternary methyl ammonium bromide, quaternary alkyl ammonium bromides, quaternary ammonium chlorides, tetraethyl ammonium hydroxide, tetraethyl ammonium chloride, tetrabutyl ammonium chloride, tetrabutyl ammonium bromide, and the like.

[031] The term “ssDNA” refers to single stranded DNA, resulting typically, but not exclusively, from denaturing double stranded DNA (“dsDNA”).

[032] The term “TE buffer” refers to the well-known buffering solution of 10mM TRIS-HCl and 1mM EDTA that is typically used in analysis of nucleic acids.

[033] The term “triamine” refers to compounds having three amino groups, including but not limited to diethylene triamine (DETA), guanidine HCl, tetramethyl guanidine, and the like.

[034] The term “nucleic acid sample” is intended to denote a sample (e.g., a composition, mixture, suspension or solution) that contains at least one nucleic acid.

[035] Unless otherwise specified, reference herein to cytosine refers to unmethylated cytosine and conversion refers to specific conversion of unmethylated cytosine to uracil.

[036] All reported temperatures are in degrees Celsius unless stated otherwise.

[037] In some embodiments, the present invention provides methods of converting cytosine to uracil in a nucleic acid sample by using a catalyzed bisulfite reaction. The methods of the present invention can provide significant benefits.

[038] The nucleic acid samples may be obtained by any conventional collection and purification process prior to use in the methods of the invention. The examples discussed below used commercially available sample lines (e.g. from Coriell or Intergen) of known methylation status, to assess the viability of the methods.

[039] Typically, the product of the reaction between the nucleic acid and bisulfite is reacted with a base to complete the conversion of cytosine to uracil. One typical base is NaOH. In some embodiments the methods herein further comprise the step of purifying the bisulfite-reacted nucleic acid prior to treatment with base. In some further embodiments, the methods further comprise the step of analyzing the product of the bisulfite conversion reaction, for example by mass spectrometry, to confirm completion of the bisulfite conversion reaction.

[040] Typical protocols in the art require the use of 3M sodium bisulfite, long reaction times of up to 16 hours, and the presence of an antioxidant. Because of the relatively high salt concentration, the low pH of the reaction and the long reaction times, the DNA can be degraded. Additionally, the ss DNA resulting from the gDNA is difficult to purify away from the high salt concentration used in the reaction. In addition, it is typically necessary to remove most of the bisulfite, which interferes with subsequent enzymatic reactions, for example those of PCR protocols. Prior procedures also require freshly prepared solutions of bisulfite and antioxidant (typically hydroquinone).

[041] Embodiments of the methods of the present invention may overcome one or more disadvantages of prior methods. For example, it has been discovered in accordance with the some embodiments of the present invention that the reaction of a nucleic acid of interest with bisulfite ion, such as magnesium bisulfite, in the presence of a quaternary amine in

accordance with the methods disclosed herein afford faster reaction times. In addition, because the reaction time is faster, less oxidation may occur. Thus, the presently disclosed methods do not require addition of an antioxidant such as hydroquinone. Additionally, magnesium bisulfite solution at 1M concentration may remain acidic in the presence of effective concentrations of polyamine catalyst (for example 0.1M DETA), whereas the corresponding solution of sodium bisulfite salt does not. Thus, methods of the invention can employ bisulfite concentrations that are significantly less than the methods known in the art, thereby affording facilitated sample preparation for PCR. Moreover, it has been discovered herein that stock magnesium bisulfite solutions can be employed, thus eliminating the need to freshly prepare those solutions. Finally, methods of the invention reduce or eliminate the need for a separate predenaturation step, and can be performed in a greatly reduced reaction volume. Thus, methods of the present invention can afford PCR yields similar to those of protocols previously known in the art, but with reduced preparation times, reaction times, and clean-up efforts.

[042] Suitable counter-ions for the bisulfite compound may be monovalent or divalent. Examples of monovalent cations include, without limitation, sodium, lithium, potassium, ammonium, and tetraalkylammonium. Suitable divalent cations include, without limitation, magnesium, manganese, and calcium.

[043] In certain embodiments, the invention comprises kits for carrying out the methods of the invention. In one embodiment, a kit of the invention includes pre-measured ingredients required for carrying out the bisulfite reaction, such as magnesium bisulfite and catalyst. In certain embodiments, the catalyst comprises tetraethyl ammonium hydroxide. In certain embodiments, the invention includes a kit containing pre-packaged materials sufficient to prepare multiple samples. In yet another embodiment, the materials will be pre-packaged with appropriate Eppendorf tubes or other reaction vessels, as appropriate.

[044] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose.

[045] The examples described herein are certain embodiments chosen to illustrate the invention. Applicant does not limit the invention to these embodiments. Rather, Applicant acknowledges that those reasonably skilled in the art will readily recognize additional variants that do not differ from the scope and spirit of the inventions disclosed herein.

EXAMPLES

[046] In accordance with the present invention, it has been found that quaternary amines, such as tetraethylammonium hydroxide (ET₄NOH), are useful catalysts in the bisulfite conversion of cytosine to uracil in nucleic acid samples.

Methyl-specific PCR Analysis

[047] Each sample discussed herein was analyzed by methyl-specific PCR (MSP). MSP provides a relatively fast analysis method for methylation status of bisulfite-treated DNA samples, providing a yes/no answer. The method is based on using primer pair sets. One primer pair is designed to anneal/PCR amplify only if all cytosines were successfully converted to uracil, and the other primer pair in the set annealed/PCR amplified if the methylated cytosine (CpG cytosines only) were methylated, and therefore not converted to uracil.

[048] The MSP pairs that amplify specific gene fragments, and the expected size of the amplicon, are the following:

[049] for the *p16* gene, unmethylated reaction (size 151):

- [050] 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense),
- [051] 5'-CAACCCCAAACCACAACCATAA-3' (antisense);
- [052] methylated reaction (size 150):
- [053] 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense),
- [054] 5'-GACCCCGAACCG-CGACCGTAA-3' (antisense);
- [055] for the *MGMT* gene, unmethylated reaction (93):
- [056] 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (sense),
- [057] 5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (antisense);
- [058] methylated reaction(81):
- [059] 5'-TTTCGACGTTTCGTAGGTTTTTCGC-3' (sense),
- [060] 5'-GCACTCTTCCGAAA-ACGAAACG-3' (antisense);
- [061] for the *DAP-kinase* gene, unmethylated reaction:
- [062] 5'-GGAGGATAGTTGGATTGAGTTAATGTT-3' (sense),
- [063] 5'- CAATCCCT-CCCAAACACCAA-3' (antisense);
- [064] methylated reaction:
- [065] 5'-GGATAGTCGGATCGAGTTAACGTC-3' (sense),
- [066] 5'-CCCTCCCAAACGCCG-3' (antisense);
- [067] for the *MLH1* gene, unmethylated reaction (124):
- [068] 5'- TTTTGATGTAGATGTTTTATTAGGGTTGT (sense)
- [069] 5'- ACCACCTCATCATAACTACCCACA (antisense)
- [070] methylated reaction (115)
- [071] 5'- ACGTAGACGTTTTATTAGGGTCGC (sense)
- [072] 5'- CCTCATCGTAACTACCCGCG (antisense)

- [073] for the *p15* gene, unmethylated reaction (154):
- [074] 5'-TGTGATGTGTTTGTATTTTGTGGTT (sense)
- [075] 5'-CCATACAATAACCAAACAACCAA (antisense)
- [076] methylated reaction (148)
- [077] 5'-GCGTTCGTATTTTGC GGTT (sense)
- [078] 5'-CGTACAATAACCGAACGACCGA (antisense)

[079] The PCR recipe used to evaluate the samples was:

2X Taq Gold PCR Master Mix	10 μ L
Fwd primer (5 μ M)	1 μ L
Rev primer (5 μ M)	1 μ L
Bisulfite treated DNA	0.5 μ L
H2O	7.5 μ L

	20 μ L

[080] 2X TaqGold PCR master mix is commercially available from Applied Biosystems. The forward and reverse primer sequences are those listed above.

[081] The following thermal cycling schedule was used:

	95 deg 5 min
40 cycles	95 deg 30sec
	60 deg 45 sec
	72 deg 1:00 min
	4 deg forever

[082] One of the primers in each set was synthesized with a 5'FAM label. A 1 μ L aliquot of the above PCR reaction was added to HiDi formamide with ROX 500 size

standard added, and denatured by heating at 95°C for 5 min. By using a FAM-labeled primer, the PCR amplicon was directly analyzed on an ABI PRISM® 310 Genetic Analyzer, with POP-4™ polymer, using run module “GS POP4 (1mL) A” (reagents and instrument all from Applied Biosystems).

[083] The presence of a PCR amplicon (i.e. a “peak”) having the correct size as observed using the 310 Genetic Analyzer indicated a successful reaction. Additionally, the height or area of the peak could be used empirically to determine how much template (i.e. bisulfite-treated gDNA) was initially present. The bigger the peak, the more DNA was initially present.

[084] The MSP-PCR product was then sometimes sequenced for further “resolution”. DNA sequencing was by standard protocol and reagents from Applied Biosystems.

[085] Prior to sequencing of the PCR amplicon, the primers and excess dNTPs used during the MSP-PCR were removed by treatment of a 4 µL aliquot of the PCR reaction with an equal volume mixture containing 2 Units each of Shrimp Alkaline Phosphatase (SAP) and exonuclease 1 (exo) (USB Corporation, Cleveland, OH). The reaction was incubated at 37°C for 1 hr, and then heat-denatured at 75°C for 15 min. A 4 µL aliquot of the exo/SAP reaction was added to a solution containing 1-4 µL of BigDye® Terminator v1.1 cycle sequencing reaction mix (Applied Biosystems), 2 µL of BigDye® Terminator v1.1 5X sequencing buffer, 2 µL of the reverse PCR primer (5 µM)(which did not have a FAM-label), and enough water for a final volume of 20 µL. Thermal cycling: 95°C/1min, 50 cycles of 96°C/10sec, 52°C/10sec, 60°C/4 min, and stored at 4°C. The cycle-sequencing reaction products were purified by an Edge Biosystems Performa® 96-well plate, dried under vacuum, dissolved in 20 µL of HiDi Formamide and analyzed on an ABI Prism 3730 DNA Analyzer with KB basecaller or a 3700 DNA Analyzer.

General Protocol for Quaternary Amine Conversion

[086] A basic protocol for the quaternary amine conversion reaction is set forth below for purposes of illustration. In an Eppendorf tube, or other suitable vessel, about 300ng DNA, 10 μ L water, 10 μ L 20% Et₄NOH (to about 0.1mM final concentration), and 85 μ L magnesium bisulfite (to about 1.3M final concentration) are combined. The resulting mixture is incubated at about 50° for 4 to 15 hours, prior to purification and subsequent PCR of the treated product. Purification can be conducted by existing means, such as in accordance with the protocol of J. Herman, *DNAs* **93**, 9821-26 (1996), incorporated herein by reference in its entirety, or with commercially available kits such as the Wizard DNA clean-up kit (available from Promega) or the EZ DNA Methylation KitTM (available from Zymo Research).

[087] A new purification method, which recovers the bisulfite-treated DNA very effectively, is the subject of the application entitled "Improved Bisulfite Method" (U.S. application Ser. No. 60/498,996 filed August 29, 2003, and also application Ser. No. 60/520,941 (5109P2) having the same title and filed concurrently herewith) each of which is hereby incorporated by reference in its entirety.

[088] In contrast with known sodium bisulfite conversion reaction protocols, all bisulfite conversion reactions disclosed herein were carried out without predenaturation, and without the inclusion of an antioxidant (e.g., hydroquinone), unless otherwise indicated.

[089] The magnesium bisulfite used in the Examples described herein was purchased as a 2M Mg (HSO₃)₂ solution from Aldrich Chemical Co., Milwaukee, Wisconsin. The pH of the solution was 2.6. The solution was used off-the-shelf, as purchased, and was not freshly prepared prior to each use.

Determination of Catalytic Effect of Quaternary Amines

[090] Examples 1-3, shown in Table 1 below, show the catalytic effect of quaternary amine compounds.

TABLE 1

Ex. 1	Ex. 2	Ex. 3
3 μ L Coriell #NA09024C	3 μ L Coriell #NA09024C	3 μ L Coriell #NA09024C
--	45 μ L water	35 μ L water
85 μ L 2 M Mg(HSO ₃) ₂	85 μ L 2 M Mg(HSO ₃) ₂	85 μ L 2 M Mg(HSO ₃) ₂
45 μ L TBAC (2M)	5.5 μ L 2M DETA	10 μ L 20% Et ₄ NOH

[091] The resultant products were purified by standard methods (Wizard kit) and analyzed by MSP using the following MSP primer set: p15M, Dapk M, MgMt M, and p16M and four corresponding unmethylated templates: p15 U, Dapk U, MgMt U, and p16 U. The methylated primer pairs, p15M, Dupk M, Mgmt M, and p16M were all negative, as expected, since the gDNA sample was not expected to be methylated. No product peak was expected or seen as with the p15M template. However, for the unmethylated gDNA samples, well-defined product peaks were seen in Et₄NOH and TBAC catalyzed reactions when the unmethylated primer pairs were used in MSP analysis.

Effect of Antioxidant and Concentration of Quaternary Amine

[092] Examples 5-8, shown below in Table 2, vary with respect to the presence or absence of antioxidant, and the identity and amount of quaternary amine.

TABLE 2

Ex. 5	Ex. 6	Ex. 7	Ex. 8
3 μ L NA 13705	3 μ L NA 13705	3 μ L NA 13705	3 μ L NA 13705
5.5 μ L 2M NaOH	5.5 μ L 2M NaOH	35 μ L water	35 μ L water

Ex. 5	Ex. 6	Ex. 7	Ex. 8
45 μ L 2M TBAC incubate at 37° for 10- 12 minutes	45 μ L 2M TBAC incubate at 37° for 10- 12 minutes	10 μ L 20% Et ₄ NOH	20 μ L 20% Et ₄ NOH
55 μ L 2M Mg(HSO ₃) ₂	30 μ L Hydroquinone	85 μ L 2M Mg(HSO ₃) ₂	60 μ L 2M Mg(HSO ₃) ₂
--	85 μ L 2M Mg(HSO ₃) ₂	--	--

[093] Each of Examples 5-8 were incubated at 50° for four hours. The reaction mixture in example 8 immediately formed a large amount of precipitate, which remained even after incubation, and was excluded from further study. Example 7 had a non-interfering amount of precipitate and was retained in the study. After the four hour incubation, the remaining three samples were purified according to the new purification method referred to above, which employed a size-exclusion purification process. The process uses a Microcon 100 (Millipore) size-exclusion device. The sample and 200 μ L of water were added to the Microcon 100 device, and the sample was then spun in the device at approximately 2800 RPM for about 8 minutes (as per manufacturers recommendation). The resultant filtrate was removed. Two subsequent washes with about 300 μ L water, each spun at about 2800 RPM for 8 minutes followed. After each, the filtrate was again removed. About 300 μ L 0.1N NaOH was added and spun at approximately 2800 RPM for about 8 minutes. Again, the filtrate was removed. After addition of about 300 μ L of water, the sample was spun in the device at 2800 RPM for about 6-8 minutes. The filtrate was removed and about 50 μ L TE buffer (approximately pH 8) was added. After about 5 minutes before it was inverted to collect the purified DNA sample in a centrifuge. Approximately 60 μ L were collected.

[094] The bisulfite-treated DNA was analyzed by MSP using the following MSP primer sets: p15 M, p15 U, Dapk M, Dapk U, Mgmt M, Mgmt U, p16 M, and p16 U. Hydroquinone did not appear to greatly enhance PCR yields. The Et₄NOH sample displayed

a far greater product peak than TBAC with or without hydroquinone. Only about 6ng of bisulfite-treated gDNA was used per PCR. Prior experiments using the published purification protocol (Wizard resin) provided much less isolated DNA based on the amount required for successful PCR. These data support the use of quaternary amine, and specifically Et₄NOH, as a catalyst.

Reduced gDNA concentration in Et₄NOH Catalyzed Magnesium Bisulfite Reaction

[095] The samples in Examples 9-12, shown in Table 3, demonstrate the bisulfite conversion of reduced amounts of DNA. These samples vary with respect to either the amount of DNA used, or reduced concentration of magnesium bisulfite/Et₄NOH.

TABLE 3

Ex. 9	Ex. 10	Ex. 11	Ex. 12
3μL Coriell 13705	1μL Coriell 13705	1μL Coriell 13705	1μL Coriell 13705
35μL water	35μL water	35μL water	35μL water
10μL 20% Et ₄ NOH	10μL 20% Et ₄ NOH	5μL 20% Et ₄ NOH	2.5μL 20% Et ₄ NOH
85μL 2M Mg(HSO ₃) ₂ ~1.3M final	85μL 2M Mg(HSO ₃) ₂ ~1.3M final	40μL 2M Mg(HSO ₃) ₂ ~1.0M final	85μL 2M Mg(HSO ₃) ₂ ~0.8M final

[096] Example 9 (3μL of Coriell) contained about 1μg DNA, and Examples 10-12 (1μL of Coriell) contains about 300ng DNA. Each of these samples was allowed to react as previously discussed, at 50°C for four hours. Subsequent to this incubation period, each was subject to the size-exclusion purification process discussed above, using the Microcon 100 device. The process differed from that previously discussed only in that slightly more water was used, and about 350μL of 0.1M NaOH was used. Each was collected in about 50μL TE buffer, and 1μL was used in subsequent PCR. Surprisingly, the 300ng sample at 1.3M magnesium bisulfite (Example 10) was observed to provide more PCR product than the 1μg sample (Example 9).

Effect of Enzyme Concentration and Template (gDNA) Concentration in MSP

[097] The same bisulfite-treated samples above, specifically, Ex. 9 and Ex. 10, were further analyzed by MSP under alternative conditions: (a) additional polymerase (TaqGold) and (b) less bisulfite-treated gDNA template. The MSP conditions are shown in Table 4, below.

TABLE 4

Ex. 13	Ex. 14	Ex. 15	Ex. 16	Ex. 17	Ex. 18
Ex. 9 bisulfite- treated DNA with xs enzyme	Ex. 10 bisulfite- treated DNA with xs enzyme	NTC with xs enzyme	1/10 of Ex. 9 bisulfite- treated DNA (0.6ng/ μ L)	1/20 of Ex. 9 bisulfite- treated DNA (0.3ng/ μ L)	1/30 of Ex. 9 bisulfite- treated DNA (0.2ng/ μ L)

[098] Each PCR was prepared as described in the MSP analysis described elsewhere herein. MSP primer sets used were Mlh M, Mlh U, Dapk M, Dapk U, Mgmt M, Mgmt U, p16 M, and p16 U. When excess TaqGold polymerase was used, an additional 2 μ L (2 units) was added to the reaction.

[099] The results show that extra enzyme in the Master Mix forced "mispriming" to occur (determined by subsequent sequencing). The 1/10, 1/20, and 1/30 dilutions of the Microcon 100 purified bisulfite-treated gDNA still provided enough gDNA template for MSP for almost all of the reactions. Successful PCR was seen even when only 0.2ng of gDNA was used in MSP. Although there were two data points that "dropped out," overall the data are excellent. Thus, it appears that as little as 0.2ng of DNA can be used with successful PCR yields.

Studies Using Additional Templates

[0100] The studies above included only unmethylated DNA. The following experiments include side by side comparisons of methylated to unmethylated gDNA. Five additional "control" reactions were evaluated with both methylated and unmethylated gDNA. These samples contained 1 μ L DNA from Coriell or 3 μ L DNA from the Intergen p16 kit (each about 300ng DNA), 35 μ L water, 10 μ L 20% Et₄NOH, and 85 μ L 2M magnesium bisulfite and heated at 50°C for about 4 hours. The samples were Coriell #34 and #35, and DNA from Intergen's p16 "kit," p16U, p16M, and a universally methylated gDNA. Each was incubated at 50° for four hours and subjected to the size-exclusion clean-up method, using a Microcon 100 filtration unit received 50mL TE. One mL was used in MSP with the following MSP primer pairs: MLH M, MLH U, Dapk M, Dapk U, Mgmt M, Mgmt U, p16M, and p16 U. Excellent MSP yields were obtained as evidence by an amplicon of the correct size, several of the PCRs were subjected to direct sequencing.

[0101] The same bisulfite treated samples were additionally analyzed by sequencing with other primers for different gene targets: E2F2, Frap, Xpd, CDKN1C, Ral GDS, Ets1, Cdh1, Apcl, Esrl, MLh1, and CMyc. These primers are:

[0102] E2F2FwdFam FAM-GGTTTGGGGAATATATTGTTGGG

[0103] E2F2Rev CTAAAAAACAACCACACTACTATTAATACC

[0100] Cdh1FwdFam FAM-TGTGTTTGTAGGAGTTTGTGTTTGTG

[0101] Cdh1Rev CTCCAAAATCCTCCAAACCC

[0102] Frap1FwdFam FAM-GATTGGTTTTTAGGGTTGGGAA

[0103] Frap1Rev TCCCCTAACCCCCCTC

[0104] XpdFwdFam FAM-GGGTTTGATTAATATTTAATTTTGGTAGG

- [0105] XpdRev TCAATCCACTAAAACACAACCAATC
- [0106] CDKN1CfwdFam FAM-GTTTTATAGGTTAAGTGTGTTGTGTT
- [0107] CDKN1Crev CACTAATACTAAAAAATCCCACAAAC
- [0108] RalGDSFwdFam FAM-
GGGTTTTATAGTTTTTGTATTTAGGTTTTTATTG
- [0109] RalGDSRev CAACTCAATAAACTCAAACCTCCCC
- [0110] ID2FwdFam FAM-
GAAGGTGAGTAAGATGGAAATTTTGTAGTA
- [0111] ID2Rev ACTAACAATTCACACACAACCTCAATCTAC
- [0112] ApclFwdFam FAM-AGGGAAAATTGGAGTAGGAGGTT
- [0113] ApclRev ACTCAACTCCCCAAAACCTATCCTTAA
- [0114] Esr1FwdFam FAM-TGGGAGATTAGTATTTAAAGTTGGAGG
- [0115] Esr1Rev CCTTAAATCTAATACAATAAAACCATCCC
- [0116] Ets1FwdFam FAM-GGGAATTTGAGATTTTTGGGAAG
- [0117] Ets1Rev CCCAACTACCAACAACATCCC
- [0118] Mlh1FwdFam FAM-GTAGTTTTTTTTTTTAGGAGTGAAGGAGGT
- [0119] Mlh1Rev CCCTACTCTTATAACCTCCCACAAAT
- [0120] CMycFwdFam FAM-GGGAGGTTATTTTGTATTATTGGG

[0121] CMycRev CCAAAACCCAAAAACAATTAACAC

Comparison of Magnesium bisulfite/ Et_4NOH to Sodium Bisulfite Protocol after 6 hour and 15 hour reaction times

[0122] A direct comparison between samples prepared according to the sodium bisulfite protocol (J. Herman, *Proc. Natl. Acad. Sci.* **93**, 9821-26 (1996)) and the magnesium bisulfite protocol as described in Ex. 10 was conducted, using Et_4NOH , NaOH, and no additive. Two plates were set up, one for a 6 hour analysis and the other for a 15 hour analysis. Both reactions took place at 50°C. Only an unmethylated gDNA sample was investigated. All reaction products were purified by the size-exclusion clean-up procedure described above and recovered in a final volume of 50 μL of TE MSP was used to analyze the converted DNA. The sodium bisulfite procedure provided gDNA that gave excellent results in MSP. The magnesium bisulfite converted gDNA gave much weaker signals in MSP than the sodium bisulfite converted DNA. However, the magnesium bisulfite/ Et_4NOH differs from the sodium bisulfite protocol in significant ways. The magnesium bisulfite/ Et_4NOH was achieved without a pre-denaturation step, much lower concentration of bisulfite, no exacting pH control, no antioxidant, and reagents were "off the shelf" and not freshly prepared.

[0123] The size-exclusion purification worked well on the sodium bisulfite samples as well as the magnesium bisulfite samples.

Direct Comparison of Magnesium Bisulfite with Et_4NOH and Sodium Bisulfite Reactions on Both Methylated and Unmethylated gDNA

[0124] The 1.3M (final concentration) magnesium bisulfite with Et_4NOH reaction was compared to the samples treated with sodium bisulfate, according to the known method.

The magnesium bisulfite recipe was 1 μ L Coriell or 3 μ L Intergen, 32 or 34 μ L water, 10 μ L 20% Et₄NOH, 85 μ L 2M magnesium bisulfite. Two methylated samples and two unmethylated samples were compared in side by side reactions with sodium bisulfite and the magnesium bisulfite. These were allowed to react for 6 and 15 hours at 50° for a total of sixteen (16) samples processed. Purification by the size-exclusion process described above was performed. In this very thorough comparison, the 16 samples, purified by Microcon 100, were all analyzed by sequencing. (The sequencing analysis allows for all cytosine in a given region to be analyzed for completeness of the bisulfite conversion to uracil.)

[0125] Eleven different primer sets for specific gene targets were used: E2F2, FRAP, XPD, CDKN, RaIDGS, IDT, CDH1, APC1, and ESR. The results showed that the methods disclosed herein are viable alternatives to the sodium bisulfite reaction. The studies herein utilized 2M magnesium bisulfite solution, which is diluted in the sample to about 1.3M. Use of a more concentrated magnesium bisulfite solution would yield higher bisulfite concentration for conversion, while still keeping reaction volumes to a minimum. Such increased bisulfite concentration in the reaction mixture could easily be employed, and would be expected to enhance PCR yields. The optimization of such reaction parameters, including volume and/or concentration of magnesium bisulfite solution, temperature, pH and other reaction conditions are expected to lead to more complete conversion, and are well within the skill of the art.

Evaluation of HPLC Model System

[0126] A model system using a synthetic, four base oligonucleotide, ATCG, was employed to determine the rate of cytosine to uracil conversion by HPLC. Samples contained Et₄NOH, no additive, tetramethyl ammonium chloride (TMAC), or guanidine HCl in the magnesium bisulfite reaction. The composition of the samples of Examples 19-22 is shown in Table 5 below.

TABLE 5

Ex. 19	Ex. 20	Ex. 21	Ex. 22
ATCG 2.5 μ L	ATCG 2.5 μ L	ATCG 2.5 μ L	ATCG 2.5 μ L
2M Mg(HSO ₃) 16.3 μ L (~1.3M final)	2M Mg(HSO ₃) 16.3 μ L (~1.3M final)	2M Mg(HSO ₃) 16.3 μ L (~1.3M final)	2M Mg(HSO ₃) 16.3 μ L (~1.3M final)
20% Et ₄ NOH 2 μ L	--	TMAC 1.25 μ L	3M Guanidine HCl 0.83 μ L (0.1M final)
Water 4.2 μ L	Water 6 μ L	Water 4.95 μ L	Water 5.4 μ L
pH ~ 4	pH ~3	pH ~3	pH ~3-4

[0127] Each sample was heated at 50° for about 28 minutes. The pH of the samples was measured after heating with pH paper, and is therefore approximate. All samples reacted comparably to each other, with the Et₄NOH reaction performing slightly better than the others.

[0128] Another HPLC study was conducted evaluating Et₄NOH, NaOH, Et₄NCl, and guanidine thiocyanide as catalysts. The composition of the samples (Examples 23-26) is shown in Table 6, below.

TABLE 6

Ex. 23	Ex. 24	Ex. 25	Ex. 26
ATCG 2.5 μ L	ATCG 2.5 μ L	ATCG 2.5 μ L	ATCG 2.5 μ L
2M Mg(HSO ₃) ₂ 16.3 μ L (~1.3M final)	2M Mg(HSO ₃) ₂ 16.3 μ L (~1.3M final)	2M Mg(HSO ₃) ₂ 16.3 μ L (~1.3M final)	--

20% Et ₄ NOH 2μL	5M NaOH 0.5μL	2M Et ₄ NCl 2μL	2M guanidine thiocyanide 16.3μL
Water 4.2μL	Water 5.7uL	Water 4.2μL	Water 6.2uL
pH ~3	pH ~3	pH ~2	pH ~4

[0129] Each sample was heated at 50° for about 32 minutes. pH was measured after heating with pH paper, and is therefore approximate. Each of the reactions compared favorably to each other, with the exception of the guanidine thiocyanide reaction, which did not react at all. The Et₄NOH reaction provided the best results, with the NaOH reaction being nearly as effective. The Et₄NCl reaction was useable, but was less effective than either the NaOH reaction or the Et₄NOH reaction. Thus, pH may be important in the reaction.

[0130] Applicants have also discovered that, although they see significant benefits in using magnesium bisulfite instead of sodium bisulfite, significant improvements may also be seen, regardless of which bisulfite is used, with the modified purification processes discussed herein. Processes for purification are further discussed in the application entitled "Improved Bisulfite Method" (U.S. application Ser. No. 60/498,996 filed August 29, 2003, and also application Ser. No. 60/520,941 (5109P2) having the same title and filed November 17, 2003), assigned to the Assignee hereof, and which is incorporated by reference in its entirety. One embodiment of that process uses a Microcon 100 (Millipore), or similar, size-exclusion device. According to one embodiment of that method, the sample and 200μL of water was added to the Microcon 100 device, and the sample was then spun in the device at approximately 2800 RPM for about 8 minutes (as per manufacturers recommendation). The resultant filtrate was removed. Two subsequent washes with about 300μL water, each spun at about 2800 RPM for 8 minutes followed. After each, the filtrate was again removed. About 300μL 0.1N NaOH was added and spun at approximately 2800 RPM for about 8 minutes. Again, the filtrate was removed. After addition of about 300μL of water, the sample was spun in the device at 2800 RPM for about 6-8 minutes. The filtrate was removed

and about 50 μ L TE buffer was added. After about 5 minutes before it was inverted to collect the purified DNA sample in a centrifuge. Approximately 60 μ L were collected.

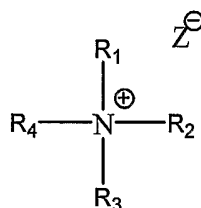
[0131] While the above-described methods of PCR and sequencing are currently preferred, they are not the only methods useable. The present invention is not limited to these any particular embodiments, or any of the examples above. Rather, other variants of these methods will be apparent to those skilled in the art and are within the scope and spirit of the invention disclosed herein.

CLAIMS

What is claimed is:

1. A method for converting cytosine to uracil comprising the steps of:
 providing a nucleic acid comprising at least one cytosine nucleobase ; and
 reacting said nucleic acid with a bisulfite ion, in the presence of a quaternary amine catalyst.
2. The method of claim 1 wherein said reacting is performed in the presence of a quaternary amine catalyst having the Formula

Formula I



or a derivative thereof, wherein:

- R_1 , R_2 , R_3 and R_4 are each independently alkyl; and
- Z is selected from halides and OH.
3. The method of claim 2 where R_1 , R_2 , R_3 and R_4 are each independently C_1 - C_4 alkyl.
4. The method of claim 1 wherein said quaternary amine catalyst comprises a quaternary ammonium.
5. The method of claim 1 wherein said quaternary amine catalyst comprises a quaternary alkyl ammonium salt.

6. The method of claim 1 wherein said quaternary amine catalyst comprises a quaternary alkyl ammonium halide.
7. The method of claim 1 wherein said quaternary amine catalyst comprises a quaternary ammonium chloride or a quaternary ammonium bromide.
8. The method of claim 1 wherein said quaternary amine catalyst comprises at least one of quaternary methyl ammonium bromide, tetraethyl ammonium hydroxide, tetraethylammonium chloride, tetrabutyl ammonium chloride and tetrabutyl ammonium bromide.
9. The method of claim 1 wherein said catalyst comprises tetraethylene ammonium hydroxide.
10. The method of claim 1 wherein said reaction is performed in a solution containing bisulfite ion in a concentration of from about 0.5M to about 2 M.
11. The method of claim 10 wherein said solution contains bisulfite ion in a concentration of about 1M to about 2M.
12. The method of claim 1 wherein said reaction is performed in a solution containing magnesium bisulfite in a concentration of from about 0.5M to about 2M.
13. The method of claim 12 wherein said solution contains magnesium bisulfite in a concentration of about 1M to about 2M.
14. The method of claim 2 wherein said reaction is performed in a solution containing magnesium bisulfite in a concentration of from about 0.5M to about 2M.

15. The method of claim 9 wherein said reaction is performed in a solution containing magnesium bisulfite in a concentration of from about 0.5M to about 2M.
16. The method of claim 5 wherein said reaction is performed in a solution containing magnesium bisulfite in a concentration of from about 0.5M to about 2.5M.
17. The method of claim 1 wherein said bisulfite solution comprises magnesium bisulfite having a concentration of at least about 1M.
18. A method for conversion of cytosine to uracil comprising the steps of:
reacting a DNA sample in solution with a bisulfite ion and quaternary amine catalyst, wherein the final concentration of bisulfite is at least from about 1M to about 3M.
19. The method of claim 18 wherein said final concentration of said bisulfite is about 1.3M.
20. The method of claim 18 wherein said reaction is performed at about 50 degrees for about 4 to about 15 hours.
21. The method of claim 18 wherein said bisulfite salt comprises magnesium bisulfite, and said quaternary amine catalyst comprises tetraethyl ammonium hydroxide.
22. The method of claim 21 wherein said reaction is performed at about 50 degrees for about 4 to about 15 hours.
23. The method of claim 1 wherein said nucleic acid is gDNA.
24. The method of claim 1 further comprising treating the product of said reaction of said nucleic acid and said bisulfite ion with a base.

25. The method of claim 24 wherein said base is NaOH.
26. A kit for use in conversion of cytosine to uracil in a nucleic acid comprising:
- magnesium bisulfite; and
- a quaternary amine catalyst.
27. The kit of claim 26, wherein said magnesium bisulfite is provided as an approximately 2M solution.
28. The kit of claim 26 wherein said quaternary amine catalyst comprises a quaternary ammonium compound.
29. The kit of claim 26 wherein said quaternary amine catalyst comprises a quaternary alkyl ammonium salt.
30. The kit of claim 26 wherein said quaternary amine catalyst comprises a quaternary alkyl ammonium halide.
31. The kit of claim 26 wherein said quaternary amine catalyst comprises a quaternary ammonium chloride or a quaternary ammonium bromide.
32. The kit of claim 26 wherein said quaternary amine catalyst comprises at least one of quaternary methyl ammonium bromide, tetraethyl ammonium hydroxide, tetraethylammonium chloride, tetrabutyl ammonium chloride and tetrabutyl ammonium bromide.
33. The kit of claim 26 wherein said quaternary amine catalyst comprises tetraethylene ammonium hydroxide.
34. The kit of claim 26 further comprising a nucleotide polymerase and one or more primers for sequencing or amplification.