HUMAN GENETIC SIGNATURES

MethylEasy™ DNA Bisulphite Modification Kit

User Guide

For Research Use Only

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1. INTRODUCTION

1.1 The DNA Bisulphite Modification Method

Prior to the early 1990s, there were very few techniques that could assess the methylation patterns in genomic DNA at the level of individual CpG sites and most of these techniques required relatively large quantities of starting DNA (up to 10 µg). This difficulty was overcome by the advent of the DNA Bisulphite Modification Method conceived by Dr G. W. Grigg and brought to practise by Dr D. Millar (now both members of Human Genetic Signatures Pty Ltd) and Dr M. Frommer (1).

The bisulphite method for determining the methylation status of cytosine residues in a DNA molecule depends on the reaction of bisulphite with cytosines in single stranded DNA. Cytosines are converted to uracils whereas 5-methylcytosines (5-mC) are unreactive (see Figure 1 and Reference 2). The modified DNA strands can be amplified using Polymerase Chain Reaction

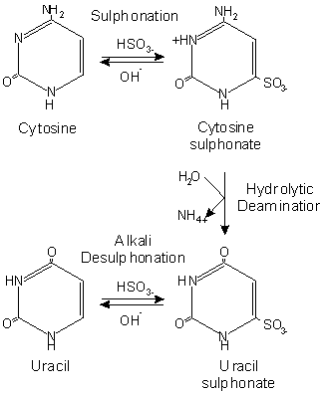

Figure 1: The DNA Bisulphite Modification Method

(PCR) and either sequenced directly, or cloned and sequenced to give methylation data from single DNA molecules.

Although 5-mC was discovered more than 50 years ago (3), its precise function and significance in the control of gene expression remained elusive for a further quarter of a century. Riggs (4), Holliday and Pugh (5) were the first to propose that methylation in mammalian DNA might have an important role in the regulation of gene expression. Their theories provided a model for the epigenetic inheritance of a given pattern of DNA methylation, and proposed a role for DNA methylation in the specific control of gene expression in given cell types. It was also proposed to account for those cases, such as X chromosome inactivation in female eutherian mammals, where only one of two homologous genes in a diploid cell is active, whilst the other is inactive.

More recently, the role of aberrant DNA methylation in disease has been the focus of much scientific interest. It has been shown that both hyper and hypo-methylation are common and are early events in the progression of cancers (6, 7). In particular, aberrant methylation in many cases has been associated with the loss of expression of the so-called tumour suppressor genes. To date, hypermethylation has been associated with over a hundred genes in cancer. In addition, hypermethylation of specific genes in cancerous cells may provide excellent early markers for cancer diagnosis (7).

Human Genetic Signatures Pty Ltd has invented a new DNA Bisulphite Modification Method which dramatically improves the yield and the efficiency of the analysis of modified DNA. The **MethylEasy**™ DNA Bisulphite Modification Kit has been developed as a result of this innovation and patent protection is being pursued by Human Genetic Signatures Pty Ltd for the **MethylEasy**™ technology. The **MethylEasy**™ methodology is pivotal for understanding the roles of DNA methylation in embryonic development, gene regulation, chromatin integrity, genomic imprinting and human diseases, especially cancer (8–15). In mammalian DNA, the main modified

base is 5-methylcytosine (5-mC), and occurs at a level of 2–5 % of all cytosine residues. This DNA modification predominantly occurs at cytosine residues that are located in CpG doublets (16).

1.2 Previous DNA Modification Methods

There are shortcomings with all previous methods of determining the methylation status of any DNA molecule. Conventional bisulphite treatments utilized to date result in the loss of up to 96% of the starting DNA (17), require restriction endonuclease digests, embedding of the DNA in agarose, multiple tube changes and column purification steps.

The **MethylEasy**™ Kit addresses all of these shortcomings and is a one tube DNA modification method that requires no DNA pre-treatment, no column purification steps, and results in virtually no loss of DNA, improved sensitivity (see Figure 2), amplification efficiency (see Figure 3), longer fragment generation and increased stability of the template DNA even at room temperature for over 1 month (see Figures 2–4). In addition, the **MethylEasy**™ protocol is rapid and simple to carry out. Furthermore, **MethylEasy**™ is easily integrated into existing technologies such as Methylation Specific PCR (18),

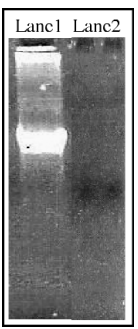

Figure 2:

Recovery of starting DNA (2µg) using **MethylEasy**™ compared to recovery using a conventional DNA bisulphite treatment, as electrophoresed on a 2% agarose gel.

Lane 1 is DNA recovered after **MethylEasy**™ modification; Lane 2 is DNA recovered after conventional bisulphite modification.

High molecular weight DNA can be seen in the sample treated with **MethylEasy**™ but not in the conventionally treated sample.

COBRA (19), MS-SNuPE (20), MALDI-TOF (21) and more recently microarray based analysis (22).

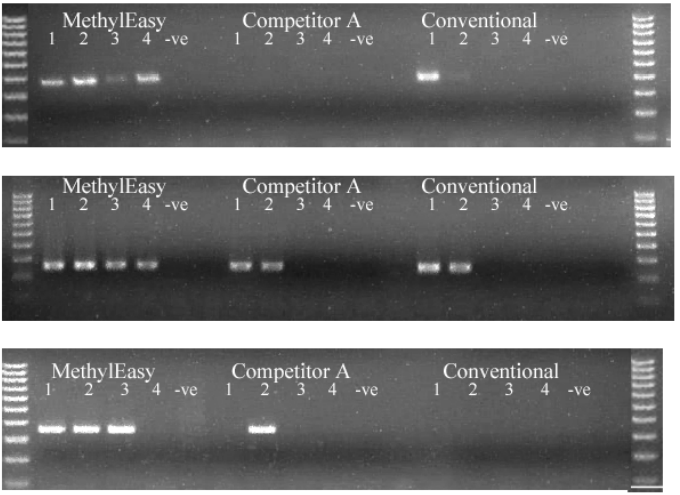

Figure 3:

Sensitivity of DNA modification technology using **MethylEasy**™ compared to bisulphite treatment using a competitor kit and conventional bisulphite treatment. Comparison is for three different genes. These genes were amplified by PCR from bisulphite treated genomic DNA. In each case:

Lane 1: 100 ng of starting DNA

Lane 2: 10 ng of starting DNA

Lane 3: 1 ng of starting DNA

Lane 4: 100 pg of starting DNA

Lane 5: No DNA control

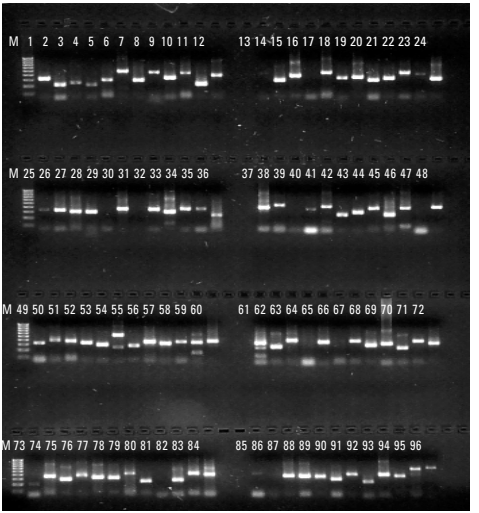

Figure 4:

Genome-wide PCR representation of 96 different loci following **MethylEasy**™ Conversion of 2 µg of DNA.

This result demonstrates the efficiency of the **MethylEasy**™ procedure over the whole genome.

Two µg of genomic DNA was extracted from human granulocytes and then subjected to **MethylEasy**™ conversion and resuspended in 100 µL of **MethylEasy**™ Reagent #3.

Ninety six (96) individual bisulphite primer sets were used for PCR amplification using 0.5 µL (10 ng) of **MethylEasy**™ converted DNA in each amplification reaction according to the **MethylEasy**™ method B. 1/10th of each amplified sample was electrophoresed on a 2% agarose gel.

M=100 bp DNA size ladder.

2. NOTICE TO CUSTOMERS

2.1 Important information

The **MethylEasy**™ kit is authorised for Research Use only and is not tested for use in diagnostic or therapeutic applications.

To discuss licensing for other applications, contact Human Genetic Signatures Pty Ltd at: <methyleasy@geneticsignatures.com>.

2.2 Intellectual Property

The **MethylEasy**™ kit and method of use is covered by pending US (10/428,310) and International patent applications.

MethylEasy™ is a trade mark of Human Genetic Signatures Pty Ltd.

2.3 Handling Precautions

Please note, reagents #1 and #2 are potential irritants. It is the responsibility of all users to consult the Material Safety Data Sheet (MSDS) before using this product. The MSDS for the **MethylEasy**™ kit is available at <www.geneticsignatures.com>. Be sure to wear gloves and avoid inhaling dust when adding Reagent #1 to the tube containing Reagent #2. After mixing of Reagents #1 and #2, store any unused material in the dark at 4°C for up to 1 month.

2.4 Contents of the MethylEasy™ DNA Bisulphite Modification Kit

Component Name	Contents
Reagent # 1	1 x 5.2 mL
Reagent # 2	1 x 2 g
Reagent # 3	1 x 3.0 mL
Reagent # 4	1 x 25 mL
Control Sample # 1	1 x 40 µL
Control Sample # 2	1 x 20 µL
Control Samples # 3A & 3B	2 x 20 µL
2 mL microcentrifuge tubes	25 x 2 mL tubes

Note: Control Samples #1, 2, 3A and 3B should be stored at –20°C upon receipt.

2.5 Materials and Equipment Required but not Supplied

> Microcentrifuge
> NaOH pellets
> Heat block or water bath (requiring temperatures of 37°C, 55°C, 72°C and 95°C)
> PCR Thermal Cycler
> 100% Isopropanol (molecular biology grade)
> 70% Ethanol (molecular biology grade)
> Mineral Oil
> Vortex Mixer
> Molecular Biology Grade Water

2.6 Optional Materials

> Carrier (Glycogen or tRNA, strongly recommended)

3. METHOD

If using MethylEasy™ for the first time, it is highly recommended that the detailed methodology in the *User Guide* be read before carrying out the bisulphite conversion method.

*Good quality DNA should be used for the conversion reaction. It is therefore recommended that DNA to be used in conjunction with **MethylEasy**™ technology is purified using Qiagen DNA mini-kit (cat# 51304). If this is not possible then the DNA should be phenol/chloroform treated before use.*

*In addition, **do not** reduce the volume of the bisulphite reagent added to the DNA sample. In-house tests have shown that reduction of the bisulphite reagent is detrimental to the reaction.*

This kit is optimised for starting DNA concentrations from 100pg* up to 4µg.
For starting DNA concentrations from 20pg up to 50ng, follow Method A.
For starting DNA concentrations from 50ng up to 4µg, follow Method B.

Using **MethylEasy**™ eliminates the need for pre-digestion of genomic DNA prior to conversion.

3.1 Preparation for both Methods A and B

1. Start with up to 4 µg DNA in 20 µL solution using one of the 2 mL reaction tubes provided.
   Note: Reagents #1 to #4 are stable at room temperature for 1 year from the date of manufacture. Once mixed, Reagents #1 and #2 are stable for up to 1 month at 4°C in the dark. If you intend using the mixed reagent on subsequent days, it is recommended that the reagent mix is overlaid with mineral oil.
   Note: In the following step, if you require smaller volumes, you can aliquot the appropriate quantities. For example, for 5 reactions, aliquot 1.04 mL Reagent #1 and 0.4 g Reagent #2.

2. For 25 reactions, add the total volume (5.2 mL) of Reagent #1 to Reagent #2 (2 g) and mix by gentle inversion.
3. Make a 3M NaOH solution (1 g NaOH pellets in 8.3 mL water). This solution must be made up fresh prior to each use.
4. Carry out the modification method as detailed in Method A or B for Control Sample #1 in parallel with the test samples. Use 5 µl of Control Sample #1 in a final volume of 20 µL and resuspend in 30 µL of reagent #3.

   * Experimental data has shown that by using efficient primers and high quality DNA, a signal can be detected with as little as 20 pg of starting DNA.

3.2 MethylEasy™ Method A (100 pg – 50 ng starting DNA)

1. Add 2.2 µl of 3M NaOH solution to 20 µl of DNA solution and mix well by pipetting.
2. Incubate at 37°C for 15 minutes.
3. Add 220 µl combined Reagent #1 and Reagent #2, mix by gentle pipetting and overlay with 200 µl mineral oil.
4. Incubate from 4–16 hours at 37°C. Ensure solution is protected from light. *Bisulphite treatment can be carried out in as little as one hour, however, reducing incubation time can result in regional non-conversion within the amplicon. Incubation times of less than 4 hours are therefore not recommended.*
5. Remove any mineral oil from the reaction tube. Alternatively, the sample may be carefully removed from beneath the mineral oil with a pipette, and transferred to a new 2 mL centrifuge tube (not provided).
6. Add 1–2 µL (20–40 µg) glycogen (Roche cat# 901 393) and mix by pipetting.
7. Add 800 µl of Reagent #4, mix well by pipetting up and down at least 10 times.

8. Add 1 mL of 100% isopropanol SLOWLY by gently adding while pipetting, then vortex for 5 seconds.
9. Incubate at 4°C for 60 minutes.
   Note: DO NOT place at –20°C as the reagents are specifically optimised for precipitation at 4°C.
10. Centrifuge for 15 minutes at 15,000 x rpm at 4°C.
11. Carefully remove the supernatant and add 0.5 mL of 70% Ethanol.
12. Centrifuge for 10 mins at 15,000 x rpm at 4°C.
13. Remove all traces of Ethanol after the last centrifugation.
14. Allow the pellet to air dry for 15 mins at room temperature.
15. Resuspend the pellet in 10 µl–20 µl of Reagent #3 (NB: For greater than 20 ng of DNA use 20 µL of Reagent #3). If you require more sample for PCR, amounts greater than 200 pg can be resuspended in up to 25 µL of Reagent #3.
16. Incubate the sample at 95°C for 30 minutes. Centrifuge once during incubation to reduce condensation.
17. The sample is now fully converted. Use 2 µL of converted sample per PCR reaction.

    [For short term storage/frequent use, freeze converted DNA at –20°C. For longer term storage (>2–3 months) aliquot and freeze at –80°C].

3.3 MethylEasy™ Method B (51 ng – 4 µg starting DNA)

1. Add 2.2 µl of 3M NaOH solution to 20µl of DNA solution and mix well by pipetting.
2. Incubate at 37°C for 15 minutes.
3. Add 220 µL combined Reagent #1 and Reagent #2, mix by pipetting up and down and overlay with 200 µL mineral oil.

4. Incubate from 4–16 hours at 37°C. Ensure solution is protected from light. *Bisulphite treatment can be carried out in as little as one hour, however, reducing incubation time can result in regional non-conversion within the amplicon. Incubation times of less than 4 hours are therefore not recommended.*
5. Remove any mineral oil from the reaction tube. Alternatively, sample may be carefully removed from beneath the mineral oil with a pipette, and transferred to a new 2 mL centrifuge tube (not provided).
6. Add 1–2 µL (20–40 µg) glycogen (Roche cat# 901 393) and mix by pipetting.
7. Add 800 µL of Reagent #4, mix well by pipetting up and down at least 10 times.
8. Add 1 mL of 100% isopropanol by gently adding while pipetting, then vortex for 5 seconds.
9. Incubate at 4°C for 30 minutes.
   Note: DO NOT place at –20°C as the reagents are specifically optimised for precipitation at 4°C.
10. Centrifuge for 10 minutes at 15, 000 x rpm at 4°C.
11. Very carefully remove the supernatant, making sure not to dislodge the pellet, then add 0.5 mL of 70% ethanol.
    Note: The pellet may not be visible after the first precipitation, therefore keep the pipette away from the expected location of the pellet when removing the supernatant. The pellet should be more visible after the addition of 70% ethanol.
12. Centrifuge for 5 mins at 15,000 x rpm at 4°C.
13. Remove all traces of Ethanol after the last centrifugation.
14. Allow the pellet to air dry for 15 mins at room temperature.
15. Resuspend the pellet in Reagent #3 to a DNA concentration of 20 ng/µL (e.g. for 1 µg, resuspend in 50 µL), up to a maximum of 100 µL volume.

16. Incubate the sample at 72°C for 60 minutes. Centrifuge once during incubation to reduce condensation. If the starting amount of DNA was >2 µg, the samples may now be diluted to 20 ng/µl using molecular biology grade water.
17. The DNA sample is now fully converted. Use 1 µL of the converted DNA sample per PCR reaction.

    [For short term storage/frequent use, freeze converted DNA at –20°C. For longer term storage (>2–3 months) aliquot and freeze at –80°C].

3.4 Internal Control PCR reaction

Genomic DNA and control PCR primers have been provided to allow for easy troubleshooting. Control Samples #1 and #2 are control DNA provided as process controls. Control sample #1 is untreated DNA with sufficient material for 8 conversion reactions. Sample #2 is bisulphite treated DNA with sufficient material provided for 20 PCR amplifications. Control samples #3A and #3B are PCR primers and may be used to check the integrity of the recovered DNA (sufficient for 10 PCR amplifications provided). These primers are designed to amplify from both human and mouse genomic DNA.

Nested PCR primers are used to further improve the sensitivity of the detection that is achieved with **MethylEasy**™ technology. The control primers are conventional bisulphite PCR primers (not MSP primers) and have been optimised for two round PCR amplification. The use of these PCR primers for single round PCR is not recommended as in most cases no visible amplicon band will be seen following agarose gel electrophoresis.

Note: This protocol is based on the use of a heated-lid thermal cycler. If such equipment is not available, overlay reactions with mineral oil.

Control reactions:

> Control Sample #1 contains untreated genomic DNA (50 ng/µL)
> Control Sample #2 contains bisulphite treated DNA (20 ng/µL)
> Control Sample #3A contains 1st round PCR primers
> Control Sample #3B contains 2nd round PCR primers

First round PCR

1. For each reaction, add 12.5 µL of PCR Master Mix (for example, Promega Master Mix) in a thin walled PCR tube.
2. Add 8.5 µL water and 2 µL of Control Sample #3A.
3. Set up sufficient tubes for each of your samples to be analysed plus Control Samples #1 and #2 as well as a 'no template' control.

Second round PCR

1. Set up an identical number of PCR tubes with 12.5 µL of PCR Master Mix, 9.5 µL water and 2 µL of control Sample #3B.
2. Freeze these tubes until required for the second round amplification step.

PCR amplification

1. Add 2 µL of the required modified DNA if prepared by Method A protocol, or 1 µL of required modified DNA if prepared by Method B protocol.
2. Add 1 µL molecular biology grade water to the 'no template' control well.
3. Run the following PCR program:

95°C/3 min	1 cycle
95°C/1 min 50°C/2 min 72°C/2 min	30 cycles
72°C/10min	1 cycle

4. Transfer 2 µL from the first round tube (template DNA) into the second round tube and repeat the PCR program detailed above.
5. Check the products by electrophoresing on a 2% agarose gel.

4. PRIMER CONSIDERATIONS

4.1 Primer Design for Bisulphite Modified DNA

- As the bisulphite treatment converts unmethylated cytosines to uracils the majority of DNA is effectively reduced to three bases (A, T and G). This decreases the complexity of DNA and can increase the incidence of primer-template interaction at 'non specific' regions. These non specific interactions are overcome by the use of a 'nested' or 'semi-nested' PCR approach.
- If you experience problems with primer design then a general guide to the basic design rules for primers can be found in Reference 23.
- If you are still having problems, Human Genetic Signatures Pty Ltd also offers a primer design service. For further information, please email your query to <methyleasy@geneticsignatures.com>.

5. APPENDIX

5.1 Methylation Specific PCR (MSP)

MSP (17) relies on designing primers in which the 3' end of each primer pair targets a specific CpG doublet, thought to be either methylated or un-methylated at that particular site. The technique has been used for the detection of circulating DNA purified from cancer patients (20).

If performing MSP using the **MethylEasy**™ kit, the following guidelines will assist in the amplification.

5.2 MSP Guidelines

- Methylation is a heterogeneous process hence under-methylation or lack of methylation at certain CpG sites can lead to misleading results. Therefore it is always best to perform standard bisulphite sequencing on a target region before proceeding to MSP to ensure the target CpG sites are fully methylated.
- Likewise, certain normal tissue samples may contain a low level of methylation. If, for example, blood is the tissue type of choice, it is advisable to sequence several normal samples before choosing the CpG sites for primer design.
- If possible, do not perform excessive numbers of PCR cycles as this can lead to the amplification of unconverted DNA resulting in false positive reactions.
- If large numbers of PCR cycles are being used, digest resultant amplicons with an enzyme such as HpaII (CCGG) to control for non-conversion.
- If real-time PCR is being used, include an unconverted probe to control for non-converted regions (24).

5.3 MSP Primer Design

As Bisulphite treatment of DNA converts unmethylated cytosines to uracils, the bulk of the DNA template is effectively reduced to three bases (A,T and G). This decreases the diversity of DNA and can increase the incidence of primer-template interaction at 'non specific' regions. This incidence is reduced by the use of a 'nested' or semi-nested approach.

5.4 Warranty

The **MethylEasy**™ product is warranted to perform as described in its labelling and literature when used in accordance with its instructions. Human Genetic Signatures Pty Ltd's sole obligation and the purchaser's exclusive remedy for breach of this warranty shall be, at the option of Human Genetic Signatures Pty Ltd, to repair or replace the products. Human Genetic Signatures Pty Ltd will not be liable for any incidental or consequential damages in connection with the **MethylEasy**™ product.

6. TROUBLESHOOTING

PROBLEMS	POSSIBLE SOLUTIONS
No PCR product was found for any sample	PCR has failed – make sure all the components were added to the PCR reaction tube and that the PCR cycle was correct.
	Confirm that the polymerase is within its storage date and that it retains its activity.
No PCR product was found for any sample except for control sample #2	Modification has failed – check that the 3M NaOH solution was fresh and that combined Reagent #1 and Reagent #2 were no older than 4 weeks. Ensure that all the steps in the modification and clean up protocols were followed.
	DNA has been lost – ensure that the DNA pellets were not lost during the washing steps. Add carrier DNA to the sample before adding the isopropanol.
	DNA was degraded during modification – check that all reagents and tubes used during the procedure were of molecular biology quality (i.e. DNase free).
	Modification was incomplete. Return the samples to 95°C or 72°C (Method A or Method B, respectively) for a further 15 minutes incubation.
	The starting DNA was not sufficiently pure. Re-purify the starting DNA using Qiagen mini-kit (Cat.# 51304). If this is not

PROBLEMS	POSSIBLE SOLUTIONS
	possible yet it is suspected that the DNA may contain impurities, it is recommended that the DNA is treated with phenol/chloroform before use.
PCR products were present only in the control reactions	Sample DNA was degraded before modification – check that the DNA has been stored/handled correctly.
	Check that the DNA concentration is not too dilute.
PCR products were present in the control reactions only when the control primers were used	PCR primers were not designed correctly – check Section 5 below for details on the primer design.
PCR products were present in all the lanes including the 'no-template' control	Check that the PCR-grade water and not the template was added to the negative control.
	Make sure that the PCR is being set up in a separate area with dedicated reagents and equipment to prevent cross contamination.
After incubation with 100% isopropanol a visible precipitate was observed.	Warm sample to room temperature for 5–10 mins, mixing by inversion every 2 mins until precipitate no longer visible. If precipitate is still visible, heat to 37°C for 5 mins, and mix by inversion until precipitate is no longer visible. Incubate at 4°C for a further 10 mins and proceed with Step 9.

7. REFERENCES

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The Polymerase Chain Reaction (PCR) process is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries. No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser by the purchase of **MethylEasy**™. Further information on purchasing licenses to practice the PCR process can be obtained from the director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

MSP is covered by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

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