HUMAN GENETIC SIGNATURES

MethylEasy™ High Throughput

DNA Bisulphite Modification Kit – *Centrifugation Method*

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. 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

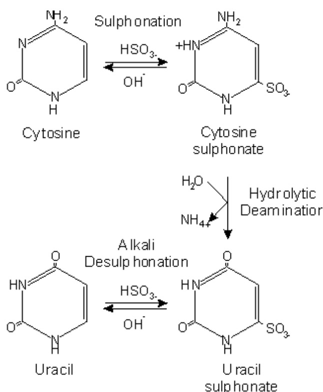

Figure 1: The DNA Bisulphite Modification Method

(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™ High Throughput** DNA Bisulphite Modification kit has been developed as a result of this patented innovation. 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 bisulphite methods used to determine 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/or column purification steps.

1.3 Advantages of the MethylEasy™ High Throughput DNA Bisulphite Modification Kit

The **MethylEasy™ High Throughput** kit addresses all of the shortcomings of previous bisulphite treatments and is in a 96 well plate format that has many advantages, including:

- no DNA pre-treatment
- no precipitation steps
- little or no loss of DNA
- improved sensitivity (see Figure 2)
- greater amplification efficiency (see Figure 3)
- longer fragment generation
- increased stability of the template DNA (at room temperature for over 1 month) (see Figures 2–4)
- 96 well plate format enables high throughput automation using a liquid handling system (a protocol has been developed using an Eppendorf epMotion 5075 automated liquid handling system. For protocol details please contact Human Genetic Signatures).

In addition, the **MethylEasy™ High Throughput** protocol is rapid and simple to carry out. Furthermore, the **MethylEasy™ High Throughput** kit is easily integrated into existing technologies such as Methylation Specific PCR (MSP) (18), COBRA (19), MS-SNuPE (20), MALDI-TOF (21) and more recently microarray based analysis (22).

Figure 2:

Recovery of starting DNA (2 µg) using **MethylEasy**™ compared to recovery using a conventional DNA bisulphite treatment, as electrophoresed on a 1% agarose gel.

Lane 1 is DNA recovered after **MethylEasy**™ modification;
Lane 2 is DNA recovered after conventional bisulphite modification (17).

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

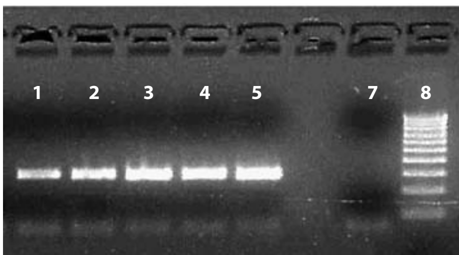

Figure 3:

Sensitivity of DNA modification technology using the **MethylEasy™ High Throughput** kit. The Lck gene product of 298 bp was amplified by PCR from bisulphite treated human genomic DNA and electrophoresed on a 2% agarose gel. All samples were resuspended in 50 µL and 2 µL was used as template for each 25 µL PCR reaction.

Lane 1: 1 ng of starting DNA
Lane 2: 10 ng of starting DNA
Lane 3: 100 ng of starting DNA
Lane 4: 1 µg of starting DNA
Lane 5: 4 µg of starting DNA
Lane 7: No DNA (negative) control
Lane 8: 100bp DNA size marker.

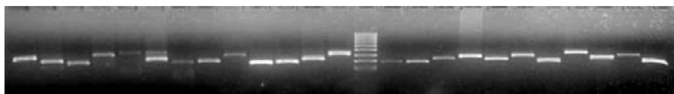

Figure 4:

PCR products from 24 different genes were amplified from a starting amount of 1 ng of human genomic DNA bisulphite treated using the **MethylEasy™ High Throughput** kit, and electrophoresed on a 2% agarose gel. DNA was resuspended in 50 µL and 2 µL of the treated DNA was used as template for each 25 µL PCR reaction.

2. NOTICE TO CUSTOMERS

2.1 Important information

The **MethylEasy™ High Throughput** kit is authorised for research use only and is not licensed 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™ High Throughput** 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 Contents of the MethylEasy™ High Throughput DNA Bisulphite Modification Kit – Centrifugation Method

Component Name	Contents
Reagent 1	1 x 20.8 mL
Reagent 2	1 x 8 g
Reagent 3	1 x 25 mL
Reagent 4	1 x 7 mL
Control Sample 1	1 x 40 µL
Control Sample 2	1 x 20 µL
Control Primers 3A & 3B	2 x 40 µL
Conversion plate	1 x 96 well
Purification plate	2 x 96 well
Wash plate	2 x 96 well
Elution plate	2 x 96 well
Clear plate sealing film for Purification plate	2 x film
Sealing caps for Conversion plate	12 x 8 caps
Sealing caps for Elution plate	8 x 12 caps

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

As a balance for the centrifuge, duplicate Purification, Wash & Elution plates are provided for use. However, Reagents supplied in this kit are sufficient for 96 samples only.

2.4 Materials and Equipment Required (not supplied)

- A centrifuge with a rotor compatible with a high clearance 96 well format plate. (In-house testing was carried out using an Eppendorf 5810).
- PCR Thermal Cycler with heated lid, compatible for 96 well format 0.2 mL low profile plates
- Oven or incubator capable of heating up to 55°C
- 80% isopropanol (molecular biology grade)
- Water (molecular biology grade)
- Carrier (glycogen [Roche Cat # 901 393] is strongly recommended) as a carrier for DNA recovery
- NaOH pellets (analytical grade)

2.5 Recommended Equipment (not supplied)

- Multi-channel pipette, up to 1 mL volume
- Multi-channel pipette, 20-200 µL
- Multi-channel pipette, 1-10 µL
- Reagent reservoirs

3. METHODS

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

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

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.

The **MethylEasy™ High Throughput** kit is optimized for starting DNA concentrations from 1 ng up to 4 µg.

Note: Incubation times and temperature will vary, depending upon the starting quantity of DNA.

3.1 Handling Precautions

Please note, Reagents 1, 2 and 3 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™ High Throughput** kit is available at www.geneticsignatures.com.

User must wear gloves and avoid inhaling dust when adding Reagent 1 to the bottle containing Reagent 2. After mixing Reagents 1 and 2, store any unused material in the dark at 4°C for up to 1 month. Reagent 3 should not be heated to high temperatures or added to acids and should be handled with gloves at all times.

3.1.1 Important Protocol Preparation

- Add the total volume of Reagent 1 to the Reagent 2 bottle and mix by gentle inversion.

Note: Reagents 1 to 4 are stable at room temperature for 1 year from the date of manufacture. However, once combined, Reagents 1 and 2 are stable for up to 1 month at 4°C in the dark.

- Make a 3M NaOH solution (eg. 1 g NaOH in 8.3 mL water). This solution must be made fresh prior to each use.
- Use 5 µL of Control Sample 1 in a final volume of 20 µL and treat in parallel with the test samples.
- Prepare the DNA you wish to convert in a final volume of 20 µL (1ng up to 4 µg).

Important Note: Ensure that Reagent 3 has not formed a solid precipitate. If so, warm the solution **(not higher than 80°C)** and mix.

3.2 Protocol

1. Add 5.0 µL of fresh 3M NaOH solution to each well of the Conversion plate.
2. Transfer 20 µL of DNA samples to each well and mix by pipetting.
3. Seal the Conversion plate with the sealing caps provided and incubate in an oven at 37°C/15 minutes.
4. Add 220 µL of the combined Reagent 1 and Reagent 2 into each well of the Conversion plate, then mix by gentle pipetting and re-seal the plate.
5. Return the Conversion plate to the oven and use either of the following conditions:
    - 1 ng–50 ng of starting DNA: Incubate at 37°C/4–16 hours
    - 51 ng–4 µg of starting DNA: Incubate at 55°C/4–16 hours

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.***

6. After incubation add the following to each well of the Conversion plate:
   - 240 µL of Reagent 3 (refer to 3.1 Important Protocol Information).
   - 20 µg glycogen (Roche Cat.# 901 393) or 1 µg tRNA.

Mix well by pipetting

Note: **As a balance for the centrifuge, duplicate Purification, Wash & Elution plates are provided for use. However, Reagents supplied in the kit are sufficient for 96 samples only.**

7. Place the Purification plate on top of the Wash plate.
8. Transfer the samples from the Conversion plate to the corresponding wells of the Purification plate, and seal with clear sealing film provided.
9. Place the Purification plate/Wash plate combination into the centrifuge and spin at 1,000 rcf at room temperature/4–5 minutes. Discard the flow-through from the Wash plate and replace.
10. Add 0.8 mL of 80% isopropanol (molecular biology grade) to each well. **Do not cover the Purification plate during the subsequent spin.**
11. Centrifuge at 1,000 rcf at room temperature/1 minute.
12. Discard the flow-through from the Wash plate and replace, then centrifuge at 1,000 rcf at room temperature/2 minutes. This step is to ensure there is no residual isopropanol in the columns as this may inhibit subsequent PCR.
13. Place the Purification plate on top of the Elution plate ensuring the tips of the Purification plate sit inside the wells of the Elution plate to prevent sample cross contamination.

14. Add 50 µL of Reagent 4 to each well of the Purification plate, placing the pipette tip close to the membrane surface without touching it.
15. Incubate at room temperature/1–2 minutes.
16. Centrifuge the combined Elution plate/Purification plate at 1,000 rcf at room temperature/1 minute.
17. Remove the Elution plate. Seal with the sealing caps provided.
18. Incubate the plate in a heated lid PCR machine using either of the following conditions:
    - 1 ng–50 ng of starting DNA: Incubate at 95°C/30 minutes
    - 51 ng–4 µg of starting DNA: Incubate at 72°C/1 hour

The DNA samples are now converted and ready for PCR amplification.

Note: Before removing the sealing caps, centrifuge the plate briefly to remove any condensation from the caps.

3.3 Internal Control PCR reaction

Genomic DNA and control PCR primers have been provided to allow for easy troubleshooting. Control Samples 1 (purple) and 2 (green) are provided as process controls. Control Sample 1 (purple) is untreated DNA with sufficient material provided for 8 conversion reactions. Control Sample 2 (green) is bisulphite treated DNA with sufficient material provided for 20 PCR amplifications. Control Primers 3A (yellow) and 3B (red) are PCR primers and may be used to check the integrity of the recovered DNA (sufficient for 20 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 rounds of 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 a heated-lid thermal cycler is unavailable, overlay reactions with mineral oil.

Control reactions:

- Control Sample 1 (**purple**) contains untreated genomic DNA (50 ng/µL)
- Control Sample 2 (**green**) contains bisulphite treated human DNA (20 ng/µL)
- Control Primers 3A (**yellow**) contains First round PCR primers
- Control Primers 3B (**red**) contains Second round PCR primers.

Control PCR

Important note

Control Primers 3A (yellow) [First round PCR] and Control Primers 3B (red) [Second round PCR] are validated 'nested' primers with sufficient volume supplied for up to 20 control PCR reactions. These primers have been supplied to facilitate the trouble-shooting process if required, and may also be used to assess the quality of your modified human or mouse DNA.

Note: The Second round PCR Reactions may be prepared in parallel with the First round PCR Reactions and frozen until required.

First round PCR

1. Set up sufficient thin-walled PCR tubes for the following reactions:
    - your converted Control Sample 1
    - Control Sample 2

- a 'no-template' (negative) control
- your own bisulphite converted DNA samples if desired, (up to 15 samples)

2. Add to each tube:
   - 12.5 µL of PCR Master Mix (*Promega Master Mix* was used in in-house testing)
   - 2 µL of Control Primers 3A
   - either 1 µL of supplied DNA **OR** 1–3 µL of your template DNA.

Do not add DNA to 'no-template' (negative) control.

   - sufficient water (molecular biology grade) to bring final volume to 25 µL.

3. Carry out the PCR according to the conditions described below, under 'PCR Amplification and Electrophoresis'.

Second round PCR

1. Set up the same number of thin walled PCR tubes as for the First round PCR.
2. To each tube add:
   - 12.5 µL of PCR Master Mix
   - 2 µL of Control Primers 3B
   - 2 µL of First round PCR product. When the quantity of starting DNA is very limited, up to 5 µL of the First round PCR product can be added.
   - sufficient water (molecular biology grade) to bring final volume to 25 µL.
3. Carry out the PCR according to the conditions described below, under 'PCR Amplification and Electrophoresis'.

PCR Amplification and Electrophoresis

1. 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

2. Products may be detected by electrophoresing on a 2% agarose gel.

3. The expected amplicon size for the Second round PCR control (using Control Primers 3B) is 240bp.

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, please refer to the general guide for the basic primer design rules, located in Reference 23.

Alternatively, Human Genetic Signatures Pty Ltd offers a primer design service incorporating our proprietary INA® technology. For further information, please email your query to methyleasy@geneticsignatures.com, or visit www.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 unmethylated 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 patterns are heterogeneous by nature and therefore cannot be assumed to be either methylated or unmethylated, at any one particular site. 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, and do not contain polymorphisms.

Certain normal tissue samples may contain a low level of 'background' 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.

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 must be used, digest resultant amplicons with an enzyme such as HpaII (CCGG) to control for non-conversion.

For real-time PCR, 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™ High Throughput** kit is warranted to perform as described in the labelling and literature when used in accordance with the supplied 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™ High Throughput** kit.

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 cycling conditions were correct.
	Confirm that the DNA polymerase used in the reaction is within its storage date and that it retains its activity.
	Amplify bisulphite converted DNA with Taq polymerase only. NB: Some proof reading enzymes do not recognise uracil.
	Residual isopropanol from the Wash step may inhibit PCR.
No PCR product was found for any sample except for Control Sample 2	Modification has failed – check that the 3M NaOH solution was freshly prepared and that combined Reagent 1 and Reagent 2 were no older than 1 month.
	Ensure that all the steps in the modification and clean up protocols were followed.
	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 for a further 15 minutes incubation as appropriate.
	Inhibitory substances could be present in the purified DNA – Increase the volume of the PCR reaction to 50 µL keeping the volume of input bisulphite treated DNA the same.

PROBLEMS	POSSIBLE SOLUTIONS
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 Sections 4 and 5 – Primer Considerations for details on primer design.
PCR products were present in all the lanes including the 'no-template' (negative) control	Check that the water (molecular biology grade) and not the template was added to the 'no-template' (negative) control.
	Ensure that the PCR is being set up in a separate area with dedicated reagents and equipment to prevent cross contamination.
Final Elution volume in wells is more than 50 µL elution volume added.	Isopropanol from the previous wash step has not been removed completely.

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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.

NOTES

HUMAN GENETIC SIGNATURES