MethylEasy™ *Xceed* Rapid DNA Bisulphite Modification Kit

User Guide
For Research Use Only.

Genetic Signatures Pty Ltd
7 Eliza St, Newtown,
NSW 2042 Australia
Phone: +61 2 9870 7580
Email: methyleasy@geneticsignatures.com
www.geneticsignatures.com

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Congratulations on your purchase of MethylEasy™*Xceed* – the next generation MethylEasy™ product.

Our original **MethylEasy™** product range revolutionised the bisulphite conversion market by introducing lossless technology where the original DNA was not degraded during the conversion process. This patented technology has set the benchmark for optimal sodium bisulphite conversions.

MethylEasy™ *Xceed* includes the following improvements:

1) 90 minute total time from wild type DNA to fully converted and purified DNA.

2) Column purification for ease of use.

3) Exceptional sensitivity with only 50 pg of starting material required – equivalent to only 8 mammalian cells.

4) Option of heat block/incubator or thermal cycler protocols for maximum flexibility.

5) Full controls are provided with each kit for absolute reaction confidence.

1. Introduction

1.1 The DNA Bisulphite Modification Method

Prior to the early 1990's, 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 Chief Scientist of Genetic Signatures Proprietary Limited) in conjunction with Dr M Frommer and colleagues (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 by the use of the Polymerase Chain Reaction (PCR) and either sequenced directly, or cloned and sequenced to give methylation data from single DNA molecules.

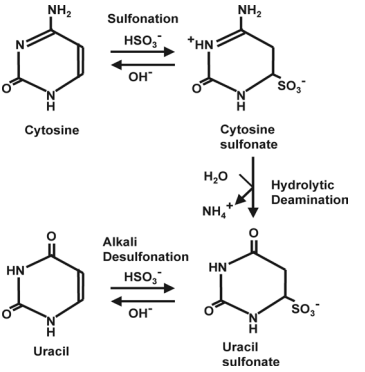

Figure 1. The DNA Bisulphite Modification Method.

Although 5-mC was discovered more than 60 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) and 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 hypomethylation 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).

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 technology has been developed as a result of this innovation and patent protection is being pursued by Genetic Signatures. The **MethylEasy™** methodology is pivotal for understanding the roles of DNA methylation in embryonic development, gene regulation, chromatin, genomic imprinting and human diseases, especially cancer (8-15). In mammalian DNA, the main modified base is 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 based methods used to determine the methylation status of any DNA molecule. Conventional bisulphite treatments utilized to date result in the loss of between 84 and 96% of the starting DNA (17), require restriction endonuclease digests, long incubation times, embedding of the DNA in agarose, multiple tube changes and/or multiple DNA purification steps.

MethylEasy™ *Xceed* addresses all of these shortcomings of previous bisulphite treatments and includes the following advantages:

- Rapid and easy to use – 90 minute total turnaround time for fully converted purified DNA
- no DNA pre-treatment
- virtually no loss of DNA (Figure 2)
- improved sensitivity (Figure 2 and 3)
- greater amplification efficiency (Figure 3)
- longer fragment generation and
- increased stability of the template DNA even at room temperature for over 1 month

In addition, **MethylEasy™ *Xceed*** is easily integrated into existing technologies such as Methylation Specific PCR (18), COBRA (19), MS-SNuPE (20), MALDI-TOF (21) and more recent methods such as microarray based analysis (22), pyrosequencing (23) and next generation sequencing (24).

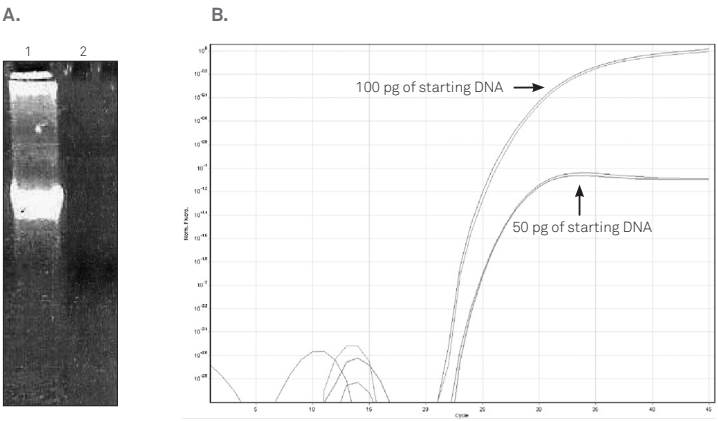

Figure 2. "Loss-less" conversion technology.

(A) Recovery of starting DNA (2 µg) using **Methyleasy™** conversion technology (Lane 1) as compared to recovery using a conventional DNA bisulphite treatment (Lane 2). The recovered product from either treatment was electrophoresed on a 2% agarose gel.
High molecular weight DNA is seen in the sample treated with **Methyleasy™** technology but not in the sample treated with conventional methods.

(B) Either 100 pg or 50 pg of DNA (16 or 8 mammalian cell equivalents respectively) was treated with **Methyleasy™*Xceed*** as per this manual and eluted in 12 µl. Nested PCR for the *Lim2* gene was performed on 4 µl of converted DNA. The second round was detected in real time on a Corbett Rotor-Gene 6000 using Syto-9 as the detection dye at a final concentration of 1 µM.

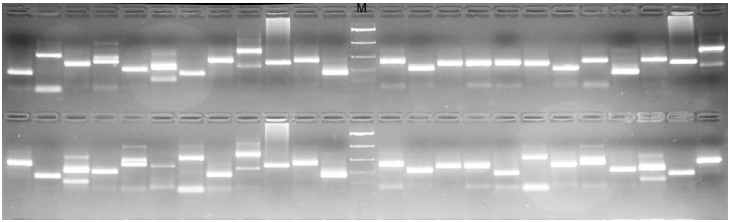

Figure 3. Sensitivity and Genomic Coverage of Bisulphite Conversion when using Methyleasy™ *Xceed*.

A total of just 50 ng of human DNA was converted as per this manual. The DNA was resuspended in 120 µL and 2 µL was seeded into 48 different PCRs that span different genomic loci. All amplifications were two rounds, and 10 µL of the second round product was electrophoresed on a 2% agarose gel.

2. Notice To Customers

2.1 Important information

The **MethylEasy™ *Xceed*** 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 Genetic Signatures at: methyleasy@geneticsignatures.com

2.2 Intellectual Property

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

MethylEasy™ is a trade mark of Genetic Signatures.

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 SDS for **MethylEasy™ *Xceed*** is available at www.geneticsignatures.com

Always wear gloves and avoid inhaling dust when adding Reagent 1 to Reagent 2. Store any unused combined Reagents 1 and 2 in the dark at 4°C for up to 4 weeks.

Reagent 3 must not be heated above 80ºC or added to acids and should be handled with gloves at all times.

2.4. Contents of the MethylEasy™ *Xceed* DNA Bisulphite Modification Kit

Component Name	Contents
Reagent 1	2 x 5.0 mL
Reagent 2	2 x 3.3 g
Reagent 3	1 x 10.6 mL
Reagent 4	1 x 5.5 mL
Reagent 5	1 x 5.0 mL
Control Sample 1	1 x 40 µL
Control Sample 2	1 x 20 µL
Control Primers 3A & 3B	2 x 40 µL
Purification Columns	40 columns
Collection tubes	40 x 2 mL tubes

Note: Control Samples 1, 2, 3A and 3B should be stored at -20°C after receipt of the kit.

2.5. Materials and Equipment Required but not Supplied

- Microcentrifuge capable of speeds of 13,000 x g
- 1.5 mL Microcentrifuge tubes
- NaOH pellets
- Heat block or water bath (requiring temperatures of 37°C, 80°C and 95°C),or PCR Thermal Cycler
- 100% Ethanol (molecular biology grade)
- Molecular biology grade water

2.6 Optional Materials

- Salmon sperm/testes DNA as a carrier when beginning with low amounts of starting material (e.g Sigma-Aldrich cat # D-9156)
- PCR thin-walled tubes with at least 0.25 mL capacity and individual lids (e.g Eppendorf cat #0030 124.359) for incubating reactions using a thermal cycler

3. Method

If using **MethylEasy™ *Xceed*** for the first time it is highly recommended that you read Section 3 in its entirety before carrying out the bisulphite conversion.

We recommend that DNA to be converted should be of good quality and be purified using Qiagen DNA mini-kit (cat# 51304). If this is not possible and you suspect the DNA may contain impurities then we recommend that the DNA is phenol/chloroform treated before use.

This kit is optimised for starting DNA amounts from 50 pg up to 5 µg.

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

3.1 Important Protocol Preparations

MethylEasy™ *Xceed* is compatible with conventional incubators or water baths, or by using 0.2 mL/0.5 mL tubes in a thermal cycler. If using a thermal cycler for the incubations it is recommended to use thin-walled tubes with individual lids and with a capacity of at least 250 µL (e.g Eppendorf tubes 0030 124.359). *Additional protocol instructions when using a thermal cycler are given in italics at the appropriate points below.*

- Turn on and set an incubator or waterbath at 80°C. If using an incubator for the bisulphite incubation then also place a tube rack into the oven to pre-heat.
- Two bottles each of Reagent 1 and 2 are provided in the kit, each sufficient for 20 reactions once combined. To perform up to 20 reactions at a time, add the entire contents of Reagent 1 to Reagent 2 and mix well by vigorous shaking. Incubate the combined Reagent 1 and 2 in the pre-heated oven or waterbath at 80°C for 15-20min, shaking twice during the incubation. If an incubator or waterbath is not available then the reagent can be dissolved by continual agitation for up to 20 minutes using a shaker or by hand. If you intend to convert less than 20 samples at a time then please see the note overleaf on how to store combined Reagent 1 and 2.

- Whilst the incubation is taking place prepare a 3M NaOH solution (e.g 100mg NaOH in 0.83 mL water). This solution must be made fresh prior to each use.
- Check Reagent 3 for any precipitate and if present warm the solution at 60°C until the precipitate dissolves.
- Add 22.1 mL of absolute alcohol to Reagent 4 before use.
- Control Sample 1 should be modified in parallel with the test samples, use 5 µL of Control Sample 1 (make up to 20 µL with water) and elute in 20 µL.
- Pre-warm Reagent 5 to 65-70°C before use.
- Before you begin, decide on the appropriate elution volume, considering the amount of starting DNA that was converted. The final elution volumes may be varied from 12-100 µL (in protocol step 16) to give a recommended final concentration of 20 ng/µL where ample DNA is available. When DNA is limiting use an elution of 12 µL and seed up to 4 µL in each PCR.

Note: All reagents are stable at room temperature up until the expiry date. However once mixed, combined Reagents 1 and 2 are stable for up to 4 weeks at 4°C in the dark, however the solution should be overlaid with mineral oil. If a visible precipitate has formed then heat the combined Reagents 1 and 2 at 80°C until all solids have re-dissolved. Be careful not to transfer any oil when adding the combined reagent to your reactions.

3.2 MethylEasy™ *Xceed* Protocol

1. Start with your DNA in a 20 µL solution in a 1.5 mL or *0.2/0.5* mL microcentrifuge tube (DNA may vary from 50 pg to 5 µg and made up to 20 µL with molecular biology grade water).
2. Add 2.2 µL of 3M NaOH solution to the 20 µL of DNA solution and mix well by pipetting.
3. Incubate the above at 37°C for 15 minutes in an incubator/water bath or thermal cycler.
4. Add 220 µL of combined Reagent 1 and Reagent 2, mix by gentle pipetting.
5. Incubate the above at 80°C for 45 minutes in the pre-heated rack in the incubator or water bath or thermal cycler. Ensure tubes are protected from light by wrapping in foil if necessary.
6. Briefly centrifuge reaction tubes in case of any condensation.
7. If starting with less than 50 ng of DNA then add 500 ng of salmon sperm/testes DNA (e.g Sigma-Aldrich D-9156) and mix well.
8. Place a purification column into a collection tube (provided).
9. Add 240 µL of Reagent 3 to the reaction mixture and mix well by pipetting. Transfer the entire solution into the column prepared in step 8.

   If using 0.2/0.5 mL tubes then first add 240 µL of Reagent 3 to a column prepared in step 8 and then immediately transfer the entire reaction solution into the column and mix well with Reagent 3 by pipetting at least 5 times.
10. Cap the columns and centrifuge for 1 min at 13,000 x g at room temperature.
11. Discard the flow-through and replace the purification column back onto the collection tube.

12. Add 0.3 mL of Reagent 4 to the column, close the cap and centrifuge for 1 min at 13,000 x g at room temperature.
    NB: Ensure that 22.1 mL of absolute alcohol has been added to Reagent 4 before use. Keep the bottle tightly closed when not in use.
13. Discard the flow-through and repeat step 12 one more time.
14. Discard the flow-through and replace the purification columns back onto the collection tubes and centrifuge again for 4 min at 13,000 x g at room temperature. Caps may be open at this step to ensure complete drying.
15. Transfer the purification column into a new nuclease free microcentrifuge tube (not provided).
16. Carefully add between 12 µL and 100 µL of pre-warmed (65-70°C) Reagent 5, onto the column membrane, without touching it, and incubate at room temperature for 1 min. Ensure that the solution is dispensed onto the column matrix and not onto the side of the column. Consider the notes in section 3.1 when determining elution volume.
17. Cap the columns and centrifuge for 1 min at 13, 000 x g at room temperature. Discard the column and cap the tube.
18. Incubate the sample at 95°C for 20 minutes in a pre-heated rack. If using a heat block, centrifuge once during incubation to reduce condensation. *The eluate may be transferred to a 0.2 mL tube for incubation in a thermal cycler.*
19. The DNA sample is now fully converted. Use 1-4 µL (20 pg to 40 ng) of the converted DNA sample per PCR reaction. If the starting amount of DNA was more than 2 µg, the samples may now be diluted to 20 ng/µL with water (molecular biology grade).

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

3.3 Internal Control PCR reaction

Genomic DNA and control PCR primers have been provided to allow for easy and complete troubleshooting. Control Sample 1 is untreated DNA with sufficient material provided for 8 conversion reactions. Control Sample 2 is bisulphite treated DNA with sufficient material provided for 20 PCR amplifications. Control Primers 3A and 3B are PCR primers specific for converted DNA 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 using **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 a heated-lid thermal cycler is unavailable, 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 Primer 3A contains First round PCR primers
- Control Primer 3B contains Second round PCR primers

Control PCR
Important note

Control Primer 3A (First round PCR primers) and Control Primer 3B (Second round PCR primers) are validated 'nested' primers with sufficient volume supplied for up to 20 control PCR reactions. These primer samples have been supplied to facilitate the troubleshooting 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 DNA samples if desired, (sufficient primers are supplied to test up to 17 of your own samples)

2. Add to each tube:

- 12.5 µL of a 2x PCR Master Mix (Adjust this volume if your mastermix is at a different concentration). Always use Taq polymerase for bisulphite converted DNA template . Hot start enzymes may be advantageous when amplifying bisulphite converted DNA
- 2 µL of Control Primer 3A
- **Either** 1 µL of supplied DNA **OR** 1–4 µL of your template DNA.
  Do not add to 'no-template' (negative) control
- Sufficient water (molecular biology grade) to bring final volume to 25 µL.
- To the 'no-template' (negative) control ONLY, add 1 µL of water (molecular biology grade)

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 Primer 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 may 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 Elecrophoresis

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/10 min	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 Primer 3B) is 240bp.

4. Appendix

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 25.

Alternatively, 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

4.2 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™ *Xceed*** kit, the following guidelines will assist in the amplification.

4.3 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 (26).

4.4 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.

4.5 Warranty

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

5. Troubleshooting

Problem	Possible Solutions
No PCR product was produced for any sample, including Control Sample 2	PCR has failed – make sure all the components were added to the tube and that the PCR cycling was correct.
	Confirm that the DNA polymerase used is within its storage date and that it retains its activity.
	Amplify bisulphite converted DNA with Taq polymerase only as some proof reading enzymes (eg Pfu) do not recognise Uracil. Similarly do not use any UNG carryover protection as this will degrade the bisulphite converted DNA.
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 was stored correctly and is no older than 4 weeks.
	Ensure that all the steps in the modification and clean up protocols were followed.
	Ensure that incubators used are operating at specified temperatures.
	DNA has been lost - add carrier DNA to the sample before adding Reagent 3.
	DNA was degraded during modification - check that all reagents and tubes used during the procedure were of molecular biology quality (ie DNase free).

Problem	Possible Solutions
PCR products were only present in Control Samples 1 and 2	Sample DNA was degraded before modification - check that the DNA has been stored/handled correctly.
	The starting DNA was not sufficiently pure. Re-purify the starting DNA using Qiagen mini-kit (Cat.# 51304). Alternatively, DNA may be purified by phenol/chloroform extraction followed by ethanol precipitation.
	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 4 for details on primer design.
PCR products were present in all the lanes including the 'no-template' (negative) 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.

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Genetic Signatures Pty Ltd
7 Eliza St, Newtown,
NSW 2042 Australia
Phone: +61 2 9870 7580
Email: methyleasy@geneticsignatures.com
www.geneticsignatures.com

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