



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

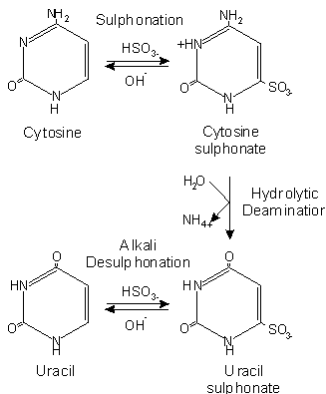


Figure 1: The DNA Bisulphite Modification Method

(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 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 column purification steps.

The **MethylEasy**[™] kit addresses all of the shortcomings of previous bisulphite treatments and 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)

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

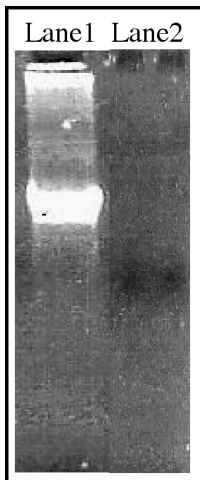


Figure 2:

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

Lane 1: DNA recovered after **MethylEasy**[™] modification;

Lane 2: 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.

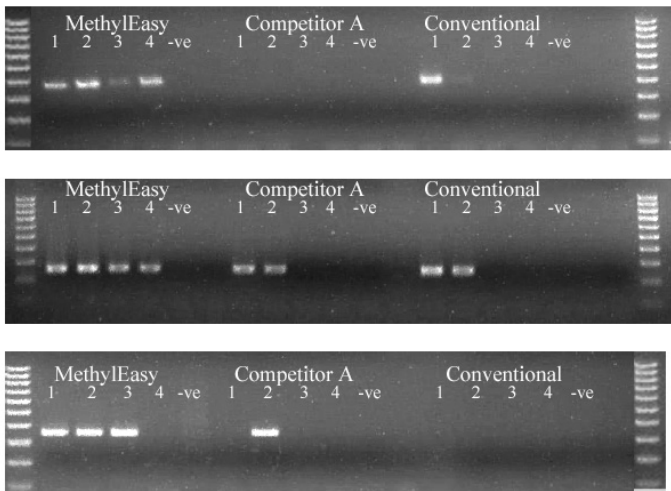


Figure 3:

Sensitivity of DNA modification technology using **MethylEasy™** compared to bisulphite treatment using a competitor kit and conventional bisulphite treatment. Three different genes were compared. These genes were PCR amplified from bisulphite treated genomic DNA and electrophoresed on a 2% agarose gel. 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

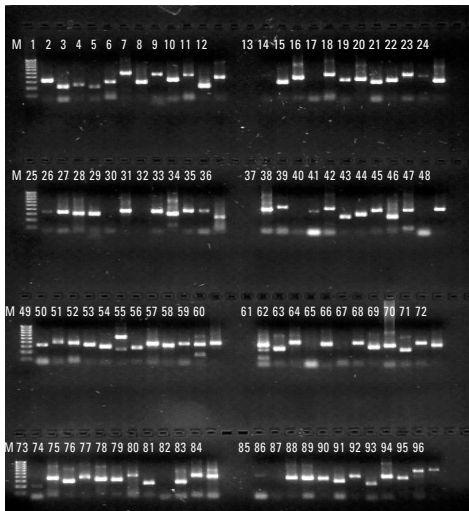


Figure 4:

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 prepared 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.genetic signatures.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 40 µL
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
- Water (molecular biology grade)

2.6 Optional Materials

- Carrier (Glycogen [Roche Cat.# 901 393] is strongly recommended as a carrier for DNA precipitation).

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.

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.

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

The **MethylEasy™** kit is optimised for starting DNA concentrations from 100 pg* up to 4 µg.

For starting DNA concentrations from 100 pg up to 50 ng, follow Method A.

For starting DNA concentrations from 51 ng up to 4 µg, follow Method B.

* In-house 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.

3.1 Important Protocol Preparation – Methods A and B

- Start with up to 4 µg DNA in 20 µL solution using a 2 mL microcentrifuge tube (provided).
- For 25 reactions, add the total volume (5.2 mL) of Reagent 1 to Reagent 2 (2 g) and mix by gentle inversion.

Note: Once combined, Reagents 1 and 2 are stable for up to 1 month at 4°C in the dark. If the combined Reagent 1 and 2 is required on subsequent days,

it is recommended that the reagent mix is overlaid with mineral oil. Reagents 1 to 4 are stable at room temperature for 1 year from the date of manufacture.

If less than 25 samples are required, smaller volumes of reagent can be used. For example, for 5 reactions, aliquot 1.04 mL Reagent 1 and 0.4 g Reagent 2.

- Make a 3M NaOH solution (1 g NaOH pellets in 8.3 mL water). This solution must be made fresh prior to each use.
- Control Sample 1 should be modified in parallel with the test samples using either Method A or B.
- Use 5 μ L of Control Sample 1 in a final volume of 20 μ L and proceed from Step 1 of the appropriate Method, A or B.
- At completion of the Method, Control Sample 1 should be resuspended in 30 μ L of Reagent 3 (at step 15 of Method A or B).

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

1. Add 2.2 μ L of 3M NaOH solution to 20 μ L of DNA solution and mix well by pipetting.
2. Incubate at 37°C/15 minutes.
3. Add 220 μ L combined Reagent 1 and Reagent 2, mix by gentle pipetting and overlay with 200 μ L mineral oil.
4. Incubate at 37°C/4–16 hours. 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 microcentrifuge 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 (for optimal results

with minimal DNA binding, use Eppendorf Safe-Lock Tubes Cat. # 0030 120.094).

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 gradual dispense and take-up, then vortex for 5 seconds.
9. Incubate at 4°C/60 minutes.

Note: DO NOT incubate 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. 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 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 at room temperature/15 minutes.
15. Resuspend the pellet in 5–20 μL of Reagent 3.
16. Incubate at 95°C/30 minutes. Centrifuge once during incubation to reduce condensation.
17. The sample is now fully converted. Use 1–3 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 20 µL of DNA solution and mix well by pipetting.
2. Incubate at 37°C/15 minutes.
3. Add 220 µL combined Reagent 1 and Reagent 2, mix by gentle pipetting then overlay with 200 µL mineral oil.
4. Incubate at 55°C/4–16 hours. 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. **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 (for optimal results with minimal DNA binding, use Eppendorf Safe-Lock Tubes Cat.# 0030 120.094).
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 by pipetting, then vortex for 5 seconds.
9. Incubate at 4°C/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 at room temperature/15 minutes.
15. Resuspend the pellet in Reagent 3 to a DNA concentration of 20 ng/μL (eg. for 1 μg, resuspend in 50 μL), up to a maximum of 100 μL volume.
16. Incubate the sample at 72°C/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 with water (molecular biology grade).
17. The DNA sample is now fully converted. Use 1–3 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 enable easy troubleshooting. Control Samples 1 and 2 are provided as process controls. 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 Samples 3A and 3B 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 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/ μ L)
- Control Sample 2 contains bisulphite treated DNA (20 ng/ μ L)
- Control Sample 3A contains First round PCR primers
- Control Sample 3B contains Second round PCR primers

Control PCR

Important note

Control Sample 3A (First round PCR primers) and Control Sample 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 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 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 Sample 3A
 - **Either** 1 μL of supplied DNA **OR** 1–3 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 Sample 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	30 cycles
72°C/2 min	
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 Sample 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**[™] product is warranted to perform as described in the labelling and literature when used in accordance with its 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**[™] 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 DNA polymerase used is within its storage date and that it retains its activity.
	Amplify bisulphite converted DNA with Taq polymerase only. N.B. Some proof reading enzymes do not recognise Uracil.
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 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.

PROBLEMS	POSSIBLE SOLUTIONS
	<p>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.</p>
<p>PCR products were present only in the control reactions</p>	<p>Sample DNA was degraded before modification – check that the DNA has been stored/handled correctly.</p> <p>Check that the DNA concentration is not too dilute.</p>
<p>PCR products were present in the control reactions only when the control primers were used</p>	<p>PCR primers were not designed correctly – see Section 5 for details on Primer Design.</p>
<p>PCR products were present in all the lanes including the ‘no-template’ (negative) control</p>	<p>Check that the water (molecular biology grade) and not the template was added to the ‘no template’ (negative) control.</p> <p>Ensure that the PCR is set up in a separate area with dedicated reagents and equipment to prevent cross contamination.</p>
<p>After incubation with 100% isopropanol a visible precipitate was observed</p>	<p>Warm sample to room temperature for 5–10 mins, mixing by inversion every 2 mins until precipitate is 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 of the appropriate method.</p>

7. REFERENCES

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NOTES

NOTES

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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