NOTE
This method seems to me a little more reliable and less cumbersome than MCA. Comment needed.

Chapter 27

Profiling DNA Methylation from Small Amounts of Genomic DNA Starting Material: Efficient Sodium Bisulfite Conversion and Subsequent Whole-Genome Amplification

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Abstract

Sodium bisulfite modification-based fine mapping of methylated cytosines represents the gold standard technique for DNA methylation studies. A major problem with this approach, however, is that it results in considerable DNA degradation, and large quantities of genomic DNA material are needed if numerous genomic regions are to be profiled. This chapter describes a method for profiling DNA methylation from small amounts of genomic-DNA starting material utilizing an efficient sodium bisulfite conversion method followed by whole-genome amplification (WGA). WGA is a useful method to overcome the problem of low initial amount of DNA and/or severe DNA degradation during conventional sodium bisulfite treatment in studies investigating DNA methylation. WGA is a relatively inexpensive process that can be optimized for high-throughput application and enables the thorough investigation of methylation at numerous genomic locations on samples for which DNA availability is low. Data from our lab has demonstrated that bisulfite-treated DNA amplified using WGA can be used for a range of downstream DNA methylation mapping procedures, including bisulfite-primer optimization, the sequencing of cloned PCR products, MS-SNuPE, and Pyrosequencing.

Key words: DNA methylation, epigenetics, sodium bisulfite, whole-genome amplification, method.

1. Introduction

Epigenetics refers to the heritable, but reversible, regulation of various genomic functions mediated through partially stable modifications of DNA and chromatin *(1)*. DNA methylation – that is,. cytosine methylation at CpG and occasionally non-CpG sites – is one of the best-understood epigenetic mechanisms and has been investigated using a myriad of laboratory techniques *(2)*. Over the last 15 years, the gold standard technique for fine

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mapping of methylated cytosines (^{me}C) has been based on the treatment of genomic DNA with sodium bisulfite, which converts unmethylated cytosines to uracils (and subsequently, via PCR, to thymidines), while methylated cytosines are resistant to bisulfite and remain unchanged *(3)*. After sodium bisulfite treatment, DNA regions of interest are amplified and sequenced to identify C→T transitions or stable C positions, respectively, corresponding to unmethylated and methylated cytosines in the native DNA. Typically, PCR amplicons are either sequenced directly to provide a strand-specific average sequence for the population of DNA molecules or cloned and sequenced to provide methylation maps of single DNA molecules *(3*, **Chapter 14***)*. An alternative approach to quantitatively assess the methylation level at specific cytosine sites within an amplicon is methylation-sensitive single nucleotide primer extension (Ms-SNuPE) using SNaPshot or Pyrosequencing *(4,5*, **Chapters 15 and 18***)*.

Conventional sodium bisulfite treatment is beset by a number of problems that result from the fact that the full conversion of unmethylated cytosines requires harsh reaction conditions which cause large-scale degradation of genomic DNA *(6)*. Alternative bisulfite-conversion protocols have been developed in which DNA is embedded in agarose during treatment to reduce DNA loss and ensure efficient conversion *(7)*, but these procedures only partially reduce the degradation and are not suited for high-throughput analysis. In many epigenetic studies the amount of genomic DNA starting material is limited, especially in experiments utilizing valuable clinical samples such as oocytes, laser-capture microdissected cells, and microscope slides. Following bisulfite treatment, converted DNA is single stranded, and prone to further degradation unless stored at −80°C. The degradation of DNA during and after sodium bisulfite treatment is a major hurdle to successful studies of DNA methylation. In addition, given the degenerate nature of sodium bisulfite-treated DNA, downstream applications such as bisulfite-PCR often require considerable optimization, further eroding valuable DNA stocks if several loci are to be interrogated.

Whole-genome amplification (WGA) methods are routinely employed on genomic DNA for genotyping and sequence analysis when the amount of starting template is extremely low. One common application of WGA, for example, is in forensic analyses where it is used to improve both the quality and quantity of DNA, and allows accurate genetic profiling from single cells *(8)*. Two commonly used WGA strategies are (i) primer extension preamplification (PEP), a *Taq* DNA polymerase PCR-based reaction first described by Zhang et al. *(9)*, and (ii) multiple displacement amplification (MDA), an isothermal genome amplification using *Phi 29* DNA polymerase *(10)*. Both methods are widely utilized

for genotyping, with several studies demonstrating the reliability of data produced from WGA templates *(11, 12)*. This chapter describes an efficient sodium bisulfite conversion method, suitable for high-throughput analyses using 96-well microtitre plates, and the subsequent application of WGA to sodium bisulfite-treated DNA.

WGA is a useful method to overcome the problem associated with a low amount of DNA-starting material and/or severe DNA degradation during conventional sodium bisulfite treatment in studies investigating DNA methylation. WGA is a relatively inexpensive process that can be optimized for high-throughput application and enables the thorough investigation of methylation at numerous genomic locations on samples for which DNA availability is low. Data from our lab has demonstrated that bisulfite-treated DNA amplified using WGA can be used for a range of downstream DNA methylation mapping procedures including bisulfite–primer optimization, the sequencing of cloned PCR products, MS-SNuPE, and pyrosequencing. While it should be acknowledged that WGA could potentially introduce biases into quantitative estimates of CpG methylation, our data suggest that such biases may not be a major problem in the profiling methods we have tested. Data from our laboratory indicates that the DNA methylation profiles obtained from WGA of sodium bisulfite-treated DNA are generally consistent with those obtained from non-WGA DNA *(13)*. Examples of direct sequencing and Ms-SNuPE analysis of WGA sodium bisulfite DNA are shown in **Fig. 27.1**. Even if not utilized for final DNA methylation data collection, given the large amount of template generated in each reaction, WGA is a useful tool for laboratories optimizing a large number of bisulfite-PCR reactions.

2. Materials

2.1. Sodium Bisulfite Treatment

1. Sterile water, preferably freshly degassed under a vacuum (*see* **Note 1**).
2. Fresh 3 M NaOH solution. Dissolve 3 g NaOH pellets in 25 mL of degassed water.
3. Fresh 0.1 M NaOH solution made from a dilution of above.
4. Fresh hydroquinone solution. Dissolve 0.22 g hydroquinone (Sigma) in 10 mL degassed water. Keep this solution shielded from light.
5. Saturated sodium bisulfite solution. Bring 10.8 g sodium bisulfite (Sigma) to 16 mL final volume in preheated degassed water (55°C). Invert to mix until solution is fully saturated.

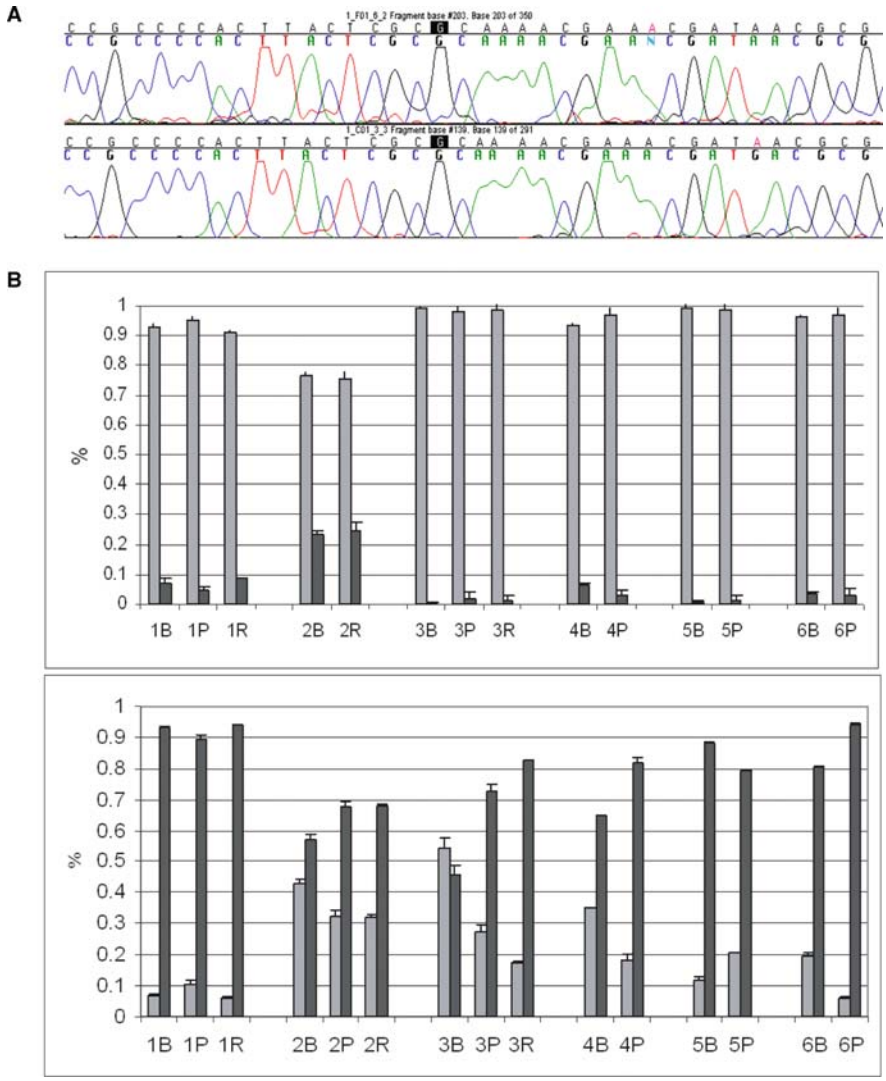

Fig. 27.1. DNA methylation profiling on WGA bisulfite DNA. (**A**) Direct sequencing chromatograms of PCR amplicons generated from normal bisulfite-treated DNA (WGA–) and PEP-amplified bisulfite-treated DNA (WGA+) from the same individual. (**B**) DNA methylation estimates obtained using Ms-SNuPE for two CpG sites on templates generated from six DNA samples (1–6). Methylation estimates derived from WGA templates are generally very similar to those produced by standard bisulfite-treated DNA templates. Light bars denote the percentage of methylated cytosines, and dark bars denote the percentage of unmethylated cytosines at each CpG site. B = non-WGA bisulfite-treated DNA; P = PEP-amplified bisulfite-treated DNA; R = bisulfite-treated DNA amplified with the Qiagen REPLI-g MDA kit.

Add 2.6 mL 3 M NaOH solution and 1.0 mL hydroquinone solution. Mix well (*see* **Note 2**).

6. Microcon YM-50 columns (Millipore) or (for high throughput) Montage PCR96 96-well filtration plate (Millipore) (*see* **Note 3**).
7. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5.

2.2. Primer Extension Preamplification (PEP) of Sodium Bisulfite DNA

1. Sterile, autoclaved water (*see* **Note 4**).
2. 100 pmol/μL 15-mer degenerate primers (5′-NNNNNNN-NNNNNNNN-3′).
3. *Taq* polymerase [5 U/μL] (NEB) or a mix of *Taq* polymerase and proofreading *Pwo* polymerase (e.g., high fidelity PCR enzyme mix [5 U/μL] (Fermentas)) (*see* **Note 5**).
4. 10 × PCR buffer.
5. 25 mM $MgCl_2$ solution.
6. 10 mM dNTP mix.
7. MiniElute PCR-purification kit (Qiagen).
8. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5.

2.3. Multiple Displacement Amplification on Sodium Bisulfite DNA (Alternative Procedure)

1. RELPI-g Mini kit (Qiagen).

2.4. Quality Control Analysis of WGA Products

1. Biotechnology-grade agarose.
2. 1 × TBE buffer: 90 mM Tris–boric acid, 2 mM EDTA.
3. 5 mg/mL Ethidium Bromide.
4. Agarose gel loading buffer.

3. Methods

3.1. Sodium Bisulfite Treatment

1. Take DNA sample (50 ng to 2 μg) and adjust volume to 10 μL (*see* **Note 6**).
2. Transfer DNA sample(s) to PCR tubes (or 96-well plate for high-throughput processing). Add 1.1 μL of fresh 3 M NaOH solution. Spin down and seal tubes (or plate).
3. Place in a thermocycler for 20 min at 42°C.
4. Spin down tubes/plate to catch condensation and carefully open seal. Add 120 μL of fresh sodium bisulfite solution, seal plate/tube with a fresh lid, invert a few times to mix, and then spin down.
5. Place in a thermocycler for 4–5 h at 55°C (*see* **Note 7**).
6. Remove plate/tubes from thermocycler, spin down, and carefully remove lid.
7. Add 100 μL of sterile water.
8. Transfer each sample to a Microcon YM-50 column (Millipore) or (for high-throughput) a well into a Montage PCR96 96-well filtration plate (Millipore).
9. Draw solution through filtration matrix by either centrifugation at maximum speed (Microcon YM-50 column) or vacuum (Montage PCR96 96-well filtration plate), until wells

are visibly empty (~4–5 min). The DNA remains on the matrix.

10. Desalt DNA by adding 175 μL of sterile water to each well and draw the solution through the matrix (via centrifugation or vacuum) as before. Repeat this step two more times.
11. Desulfonate by adding 175 μL fresh of 0.1 M NaOH. Draw solution through the matrix (via centrifugation or vacuum).
12. Perform a final wash step by drawing 175 μL of sterile water through the matrix.
13. Recover DNA by adding 50 μL of 1 × TE Buffer and incubate for 2 min at room temperature.
14. If using Microcon YM-50 columns, carefully vortex the Microcon unit for 30 s, separate sample reservoir from filtrate cup and place sample reservoir upside down into a new vial. Spin for 3 min at 1,780*g* in invert spin mode to elute DNA. If using a Montage PCR96 96-well filtration plate, use a plate shaker to release DNA from filtration matrix for 10 min at 500 rpm. Remove eluted DNA solution from individual wells using a pipette and filter tips.
15. Remove an aliquot for WGA if needed. Otherwise, store bisulfite-treated DNA at −20°C (or −80°C for long-term storage).

3.2. Primer Extension Preamplification of Sodium Bisulfite DNA

1. PEP reactions should be setup on ice, taking care to maintain a clean and sterile laboratory environment to ensure that no contamination occurs (*see* **Note 8**).
2. Each PEP reaction uses 2-μL sodium bisulfite template (from the 50 μL final volume eluted in the protocol described above). Assuming sodium bisulfite conversion was performed on 250 ng starting genomic DNA, this corresponds to ~10 ng original genomic DNA template (*see* **Note 9**).
3. Each PEP reaction is performed in triplicate to increase the likelihood that all genomic regions are adequately enriched. The total volume of sodium bisulfite DNA required for each PEP-based WGA reaction is 6 μL (corresponding to ~30 ng of the original genomic DNA template assuming bisulfite conversion was performed on 250 ng) (*see* **Note 9**).
4. Add bisulfite DNA to reaction well. Remember to add appropriate negative control samples. Use filter tips and pipette carefully. Be careful to avoid aerosol contamination.
5. Make up a master mix of the PEP amplification reagents, mix well, and aliquot 48 μL (to a total volume of 50 μL) to each bisulfite-treated DNA sample. Each 50 μL reaction contains 2 μL 100 pmol/μL PEP primer, 2 μL 10 mM dNTPs, 5 μL 10 × PCR buffer, 10 μL 25 mM $MgCl_2$, 0.8 μL *Taq* polymerase (NEB) or high fidelity PCR enzyme mix (Fermentas), and 28.2 μL sterile H_2O (*see* **Note 10**).

6. Carefully seal the PCR tubes and centrifuge the reaction mix to collect it at the bottom of the reaction well.
7. Transfer samples to a thermalcycler, preprogrammed to amplify using 50 cycles, with each cycle consisting of a denaturing step for 1 min at 95°C, a annealing step for 2 min at 37°C, a programmed ramping step of 10 s/°C up to 55°C, and an incubation step for 4 min at 55°C.
8. Following amplification, ensure the reaction is cooled to 4°C (to avoid condensation on the lid) and centrifuge to collect the product at the bottom of the PCR tube.
9. Carefully open the PCR tube/plate ensuring that no cross-contamination of wells occurs (*see* **Note 11**).
10. Carefully combine the three replicates for each sample into one tube (→150 μL total volume) and mix well.
11. (Optional – *see* **Note 12**). Add 1,000 μL of Buffer EB (Qiagen) and mix. Transfer 600 μL to a labeled MinElute column (Qiagen) and centrifuge at maximum speed for 1 min. Discard flowthrough and repeat step with the remaining 600 μL. Discard flowthrough. Add 750 μL of Buffer PE (Qiagen) and centrifuge at maximum speed for 1 min. Discard flowthrough and place the MinElute column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed. Place the MinElute column in a clean 1.5-mL microcentrifuge tube. Add 100 μL of prewarmed (~50°C) 10 mM Tris–Cl, pH 7.5 and centrifuge for 1 min. Repeat this step with another 100 μL Tris–Cl, pH 7.5.
12. Take a 5-μL aliquot for agarose gel analysis and quantification (**Section 3.4**).
13. Remaining amplified template can be kept at 4°C (for short-term storage) or −20°C (for long-term storage).

3.3. Multiple Displacement Amplification on Sodium Bisulfite DNA Using the Qiagen REPLI-g Mini Kit (Alternative Procedure to 3.2)

1. Preheat incubator or water bath to 30°C.
2. Thaw REPLI-g Mini DNA Polymerase on ice, and all other components at room temperature (*see* **Note 13**).
3. Prepare sufficient Buffer D1 (denaturation buffer) and Buffer N1 (neutralization buffer) for the total number of WGA reactions.
4. Add 5 μL of bisulfite-treated DNA (corresponding to 50 ng original genomic DNA template) to a microcentrifuge tube.
5. Add 5 μL Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly.
6. Incubate the samples at room temperature (15–25°C) for 3 min.
7. Add 10 μL of Buffer N1 to the samples. Mix by vortexing and centrifuge briefly.
8. Make a master mix comprising for each sample to be amplified including 29 μL of REPLI-g Mini Reaction Buffer and

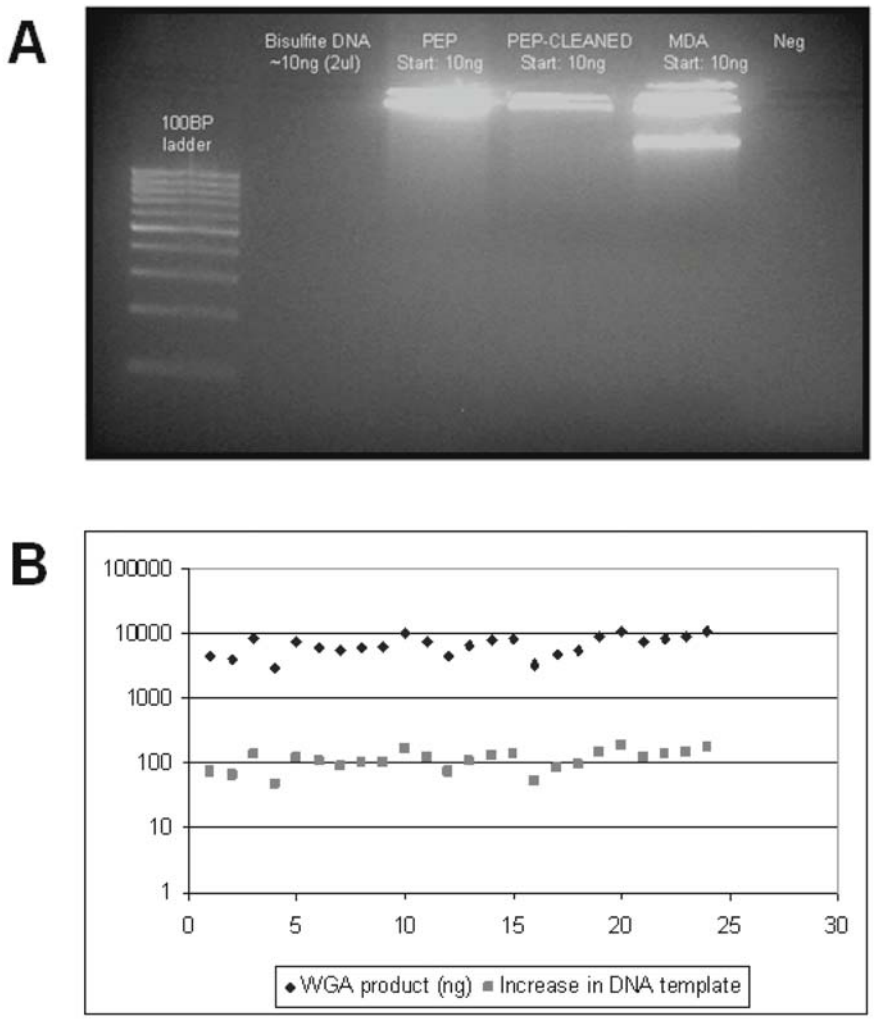

Fig. 27.2. Whole genome amplification (WGA) typically results in a large increase in bisulfite-treated DNA template. Representative WGA products obtained from standard sodium bisulfite-treated DNA starting material. Products in lanes 3 and 4 [primer-extension preamplification (PEP)] and lane 5 [multiple displacement amplification (MDA)] were produced from approximately 10 ng original bisulfite-converted DNA.;10 ng non-WGA bisulfite-treated DNA is not visible (lane 2), but both WGA methods produce clearly visible DNA smears of high molecular weight, demonstrating efficient amplification. Marker (lane 1) is the GeneRuler^{TM} 100-bp DNA ladder (Fermentas, Hanover, MD). (**B**) Typical results from spectrophotometric analysis of 25 representative PEP-amplified products. The average yield from the pooled PEP replicates was 6.8 μg (± 2.2 μg). Assuming no degradation during the sodium bisulfite conversion reaction, this represents a 113-fold (± 37) increase from the initial starting genomic DNA (60 ng).

1 μL of REPLI-g Mini DNA Polymerase, and add 30 μL of this master mix to each DNA sample.

9. Incubate at 30°C for 16 h.
10. Inactivate REPLI-g Mini DNA Polymerase by heating the sample for 3 min at 65°C.
11. Take a 5-μL aliquot for agarose gel analysis and quantification (**Section 3.4**).

12. Store amplified DNA at either 4°C (for short-term storage) or −20°C for long-term storage.

3.4. Quality Control Analysis of WGA Products

1. Before using WGA products for downstream bisulfite-PCR, it is advisable to check that the amplification reaction has worked efficiently and that there is no contamination present in the reaction. Make a 1% agarose gel by adding 1.0 g biotechnology grade agarose to 100 mL of 1 × TBE buffer and microwaving the mixture for ~2 min. Allow the gel to cool for a few minutes and add ethidium bromide to a final concentration of 5 μg/mL. Mix well. When the gel is cool, it can be poured into a small gel tray and allowed to polymerize.
2. Add 1 μL of gel loading buffer to 5 μL of each WGA-enriched sample, and load each into a separate well on the gel. Include the negative control WGA products, and also a suitable DNA size marker in the first well on the gel.
3. Allow products to run for a suitable distance on the gel and then visualize under UV light. You should observe clearly visible DNA smears of high genomic weight, demonstrating efficient amplification. The negative sample should not have such a smear (**Fig. 27.2A**).
4. Spectrophotometric analysis can be performed on the samples to determine the average yield from each reaction. **Fig. 27.2B** illustrates spectrophotometric readings from 25 representative PEP-amplified products generated in our laboratory. The average yield from pooled PEP reactions (performed in triplicate) was 6,767 ng (± 2,206 ng). Assuming no degradation during the sodium bisulfite conversion reaction, this represents a 113-fold (±37) increase from the initial starting genomic DNA (60 ng) for these samples.

4. Notes

1. Free oxygen in water can reduce the efficiency of sodium bisulfite conversion.
2. It is possible that the sodium bisulfite will not entirely dissolve. If any substrate remains, centrifuge solution before use and use the supernatant.
3. The use of the Montage PCR96 96-well filtration plate (Millipore) for high-throughput sample processing requires a suitable vacuum manifold.
4. It is imperative that all reagents used for WGA are clean and sterile – the WGA process will amplify any DNA in solution, even when present at very low concentration.
5. While we do not observe too many problems using standard Taq polymerase, the proofreading Taq polymerase mix improves the error rate inherent in WGA.

6. In our experience, the optimal starting amount of genomic DNA is between 250 ng and 1 μg. Small amounts of DNA (e.g., 50 ng or below) may not provide enough useable template for direct use following sodium bisulfite treatment, but can be used for DNA methylation profiling following WGA of the sodium bisulfite-treated DNA.
7. It can be beneficial to ramp the reaction up to 95°C for 1 min every hour to ensure that the DNA remains single stranded.
8. PEP amplification is highly efficient, and contamination problems can be an issue. For this reason, we suggest including always a negative water control in each set of WGA reactions. Use the products of these as a negative control for all subsequent PCR amplifications. Note well: even contamination from the researcher performing the PEP experiments can be a problem.
9. If this exceeds the amount of available template, it is better to do three replicates on smaller amounts of template than one replicate on a larger amount of template.
10. The high fidelity PCR enzyme mix contains a proofreading polymerase in addition to *Taq* and is thus less prone to sequence errors, but is considerably more expensive. The choice of enzyme depends upon your ultimate downstream application of the WGA material – occasional sequence errors may be less important if the products are to be used only for assay optimization, etc.
11. If possible, these postamplification steps should be performed in a separate room (or fume hood) away from the location of preamplification steps. The release of post-WGA aerosols, even in minute quantities, can lead to contamination problems that are hard to rectify.
12. This step removes excess degenerate primers and deoxynucleotides that can interfere with certain downstream applications if not removed fully. One downside is that some loss of amplified template may occur due to the maximum binding capacity of the Qiagen columns. If this is an issue, then the sample can be split and extra columns utilized.
13. The REPLI-g mini reaction buffer may form a precipitate after thawing. The precipitate can be dissolved by vortexing for 10 s.

References

1. Henikoff, S., Matzke, M. A. (1997) Exploring and explaining epigenetic effects. *Trends Genet* 13, 293–295.
2. El-Maarri, O. (2003) Methods: DNA methylation. *Adv Exp Med Biol* 544, 197–204.
3. Clark, S. J., Harrison, J., Paul, C. L., et al. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 22, 2990–2997.
4. Schumacher, A., Kapranov, P., Kaminsky, Z., et al. (2006) Microarray-based DNA methylation profiling: technology and applications. *Nucleic Acids Res* 34, 528–542.

5. Tost, J., El Abdalaoui, H., Gut, I.G. (2006) Serial pyrosequencing for quantitative DNA methylation analysis. *Biotechniques* 40, 721–726.
6. Grunau, C., Clark, S.J., Rosenthal, A. (2001) Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* 29, e65
7. Olek, A., Oswald, J., Walter, J. (1996) A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res* 24, 5064–5066.
8. Ballantyne, K.N., van Oorschot, R.A., Mitchell, R.J. (2007) Comparison of two whole genome amplification methods for STR genotyping of LCN and degraded DNA samples. *Forensic Sci Int* 166, 35–41.
9. Zhang, L., Cui, X., Schmitt, K., et al. (1992) Whole genome amplification from a single cell: implications for genetic analysis. *Proc Natl Acad Sci USA* 89, 5847–5851.
10. Dean, F.B., Hosono, S., Fang, L., et al. (2002) Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci USA* 99, 5261–5266.
11. Kuivaniemi, H., Yoon, S., Shibamura, H., et al. (2002) Primer-extension preamplified DNA is a reliable template for genotyping. *Clin Chem* 48, 1601–1604.
12. Hosono, S., Faruqi, A.F., Dean, F.B., et al. (2003) Unbiased whole-genome amplification directly from clinical samples. *Genome Res* 13, 954–964.
13. Mill, J., Yazdanpanah, S., Guckel, E., et al. (2006) Whole genome amplification of sodium bisulfite-treated DNA allows the accurate estimate of methylated cytosine density in limited DNA resources. *Biotechniques* 41, 603–607.