

Bisulfite sequencing of very small samples (PROT37)



Petr Svoboda

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic
Videnska 1083
142 20 Prague 4, Czech Republic

Email feedback to:

svobodap@img.cas.cz

Last reviewed

: 18 Oct 2007 by Jörn E. Walter, Saarland University, Postbox 151150, 66041 Saarbrücken, Germany,
j.walter@mx.uni-saarland.de

Introduction

(based on Olek et al., 1996, Schoenherr et al., 2003)

DNA methylation is a stable epigenetic mark, which can mediate gene silencing. Bisulfite sequencing allows for precise identification of methylated cytosines within DNA (Frommer et al. 1992). This method is based on different rate of chemical conversion of methylated and non-methylated cytosines to uracil (Figure 1) where non-methylated cytosines are converted efficiently while methylated cytosines remain non-reactive. This method was further developed by embedding analyzed DNA into an agarose bead (Olek et al. 1996). The protocol presented here was further optimized for bisulfite sequencing of small samples where the starting material was a small number of cells (Fedoriw et al. 2004; Svoboda et al. 2004). The smallest amount of material from which several unique clones were recovered was 25 oocytes, which corresponds to 100 DNA molecules in the initial material (Svoboda et al. 2004). This protocol can be also used for analyzing up to 200ng purified genomic DNA in one sample

Procedure

Primer design

Good primers are absolutely critical for successful bisulfite sequencing. Use genomic DNA sequence from the region you want to amplify (It sounds obvious but it is not uncommon that people accidentally design primers for cDNA sequence).

Convert genomic DNA in silico:

1. Open sequence file (FASTA format) in Word
2. Edit/Replace - replace CG with XY
3. Edit/Replace - replace C with T
4. Edit/Replace - replace XY with CG
5. Use this sequence to design primers

Design primers – We manually select primer sequences to amplify a region of interest and then we check the T_m and other parameters in the Vector NTI. Primers are usually ~ 28-30 nt long and they have T_m approx. 55 °C (comment 1). Choose primers such that they contain 5-8 G's and try to choose primers that have higher sequence complexity. Try to avoid CpG's in primer sequences – if you have no choice, use degenerated nucleotide in that position. Also, try to avoid having very T-rich sense and A-rich antisense primers, which would pair too much. If there is no other choice, you can accept $-\Delta G$ up to approx. -2.0 if it is a single strong pairing in the middle or at the 5' end of the primer and it does not provide a good template for Taq pol (which could inactivate this primer pair). Keep the PCR product under 600 bp (ideally 300-350 bp).

Day 1 (if your starting material is DNA go to day 2)

1. Prepare 2% LMP Agarose in pure water. Melt it in the microwave, mix on magnetic stirrer (note 1).
2. Add 20 μ L of melted agarose to cells placed at the bottom of a 2 mL Eppendorf tube, spin briefly down (note 2).
3. Overlay with 300 μ L of mineral oil and incubate for a few minutes at 65 °C (note 3).
4. Chill on ice for 2 minutes to solidify
5. Add 800 μ L of Lysis buffer to the bead.
6. Add 2 mL of Proteinase K per sample (40 mg per reaction – stock 20 mg/ml)

7. Incubate overnight at 50 °C for 12 to 14 hours, with gentle shaking if possible ([note 4](#)).

Day 2

1. Wash the bead 3 times with 1 mL of TE (10 mM Tris-HCL, pH 7.6, 1 mM EDTA) for 15 minutes with gentle mixing ([note 5](#)).

If you are using genomic DNA start here:

- Add 30 µL of melted 2% [LMP Agarose](#) to DNA (up to 200 ng in up to 4 µL)
 - Overlay with 300 µL of paraffine oil and incubate for a few minutes at 65 °C
 - Chill on ice for 2 minutes to solidify
2. Add 500 µL of 0.3M NaOH and incubate for 15 min, remove NaOH solution.
(*denaturation of DNA strands*)
 3. Repeat incubation with 500 µL of 0.3M NaOH for 15 min, remove NaOH solution.
 4. Incubate with 500 µL of 0.1M NaOH for 10 min, remove NaOH solution.
(*to solidify agarose beads*)
 5. In the meantime start preparing fresh [10 mM hydroquinone](#) and [40.5% Sodium bisulfite](#) in pure water ([note 6](#)). Both solutions are light sensitive, so try to avoid any unnecessary exposure to light (wrap Falcon tubes with aluminum foil, cover beaker with dissolving sodium bisulphate etc.).
 6. Transfer the bead into a new 2 mL Eppendorf tube:
 - Cap the tube containing the bead with a cap of a new tube, invert and tap on the tube with the bead – the bead drops on the cap.
 - Then release carefully the cap with the bead, close the new tube and tap on it to place the bead at the bottom. This step prevents diluting [LMP Agarose](#) and increasing bead size in subsequent steps.
 7. Overlay the bead with 300 µL of mineral oil, heat to 80 °C for 15 minutes.
(*full separation of individual DNA strands*)
 8. Resolidify the agarose bead by chilling on ice (at least 2 minutes).
 9. Set up the bisulfite reaction. Add 15 µL of 10 mM hydroquinone and 255 µL of [40.5% Sodium bisulfite](#)
 10. Incubate at 50 °C for 4-6 hours with gentle mixing (e.g. Eppendorf Thermomixer at 400 rpm), cover with aluminum foil to protect samples against light ([note 7](#)).
 11. Stop the bisulfite reaction by removing solutions and mineral oil and wash the bead with 1 mL of TE for 15 minutes.
 12. Repeat wash 5 times with TE. If necessary, you can store the bead in TE after the second wash at 4 °C overnight and continue the next day
 13. Incubate the bead with 500 µL of 0.2 M NaOH for 15 minutes at room temperature, remove NaOH.
 14. Incubate the bead with 500 µL of 0.2 M NaOH for 15 minutes at 37 °C. (*desulfonation step*)
 15. Neutralize NaOH with 100 µL of 1M hydrochloric acid.
 16. Briefly wash the bead with 1 mL of TE.
(*pH is often not neutral after HCl neutralization, this step assures proper neutralization*)
 17. Wash twice with 1 mL of pure water, 15 minutes each.
 18. After removing the last wash, transfer the bead into a new Eppendorf tube.
 19. Add pure water to the final volume of approx. 100 µL. Heat the tube at 80 °C for 5 min, mix briefly by vortexing and use aliquots of diluted melted agarose for PCR reaction ([note 8](#)).

Suggestions for PCR, cloning and sequencing of PCR products

PCR

For very small samples: to assure detection of as many individual strands as possible we recommend to run more PCR reactions for one locus (four to ten) and then clone individual amplicons separately. If one uses a single PCR reaction for a very small sample, there is a high risk of a strong clonal effect – i.e. a large fraction of sequenced clones will be derived from a single converted template molecule. If the methylation pattern is homogeneous, one cannot distinguish between identical sequences stemming from the same single DNA molecule and sequences stemming from different molecules with the same pattern. This problem can be addressed if one generates independent amplicons and clones them separately. Identical sequences cloned from different amplicons give a better idea about the distribution of DNA methylation.

When starting with 200 ng of genomic DNA, clonal effects are relatively small, so one PCR reaction for each locus is typically sufficient. We usually use 1/10 of a melted bead in 50 µL PCR reaction. If there is a suspicion of clonal effects we suggest to run more individual PCRs on the rest of the sample.

When setting up PCR, we melt the sample in the Eppendorf Thermomixer (800 rpm) while preparing PCR mastermix. Then we divide diluted melted agarose into PCR tubes containing the rest of the PCR reaction mixture. It is recommended to use a hot-start PCR to avoid nonspecific primer amplification ([comment 2](#)). We use Amplitaq GOLD polymerase, which requires heat activation (15 minutes at 94 °C).

We typically use 20-25 μL of diluted melted agarose in 100 μL PCR for very small samples (20-50 oocytes) and 10 μL of diluted melted agarose in 50 μL PCR for genomic DNA ([comment 3](#)). To further improve specificity of amplification, we use touch-down PCR. The following program works well for primers with $T_m \sim 55^\circ\text{C}$

94 $^\circ\text{C}$ for 15 min

94 $^\circ\text{C}$ for 30 sec

62- \rightarrow 55 $^\circ\text{C}$ for 30 sec - 14 cycles with a gradual decrease of T_m 0.5 $^\circ\text{C}$ per cycle

72 $^\circ\text{C}$ for 1 min

94 $^\circ\text{C}$ for 30 sec

55 $^\circ\text{C}$ for 30 sec - 36 cycles

72 $^\circ\text{C}$ for 1 min

72 $^\circ\text{C}$ for 15-20 min - 1 cycle

It is possible to sequence the PCR product directly but we do not recommend it as it yields misleading results. The main reason is that the height of peaks in the sequencing chromatogram is extremely biased because of the biased nucleotide content of the sequence. In some experiments direct PCR product sequencing can produce a completely different results compared to the analysis of individual clones ([figure 2](#)). Therefore, for direct analysis of PCR products one should use an appropriate quantitative approach such as, for example, pyrosequencing ([comment 4](#)).

PCR cloning:

It is possible to use completed PCR reaction directly for cloning but we prefer to purify amplicons via gel extraction. We run 50 μL of PCR reaction in 1.2% agarose gel (regular agarose, in 1x TAE or 0.5 x TBE), gel extract amplicons (Qiagen Gel extraction kit, final elution volume 30 μL) and use 4 μL for TOPO TA cloning (Invitrogen, TOPO TA cloning kit – dual promoter). As noted above, if you work with small samples, it is better to clone each amplicon separately. Use blue-white selection for TOPO TA cloning.

Pick at 5-10 colonies from each plate for miniprep. Submit for sequencing (SP6 primer is the best for pCR II plasmid – for some reason the PCR product tends to be cloned into the vector in one orientation (antisense with respect to the pCR II sequence). Sequencing of GT rich regions is often very difficult and because of the bias in cloning orientation, the SP6 primer usually sequences the easier-to-sequence CA-rich strand).

Sequence analysis:

We use Bioedit (freeware, operates in Windows systems) to generate alignment of analyzed sequences. Bioedit can upload batches of sequences and it also recognizes original ABI sequencing files. Although Bioedit can perform multiple sequence alignment, we prefer to do the alignment for smaller sets manually to make sure we appropriately inspect all suspiciously looking nucleotides (Ns, nucleotide insertions etc.). We recommend visual inspection of sequencing chromatograms to verify identified methylated cytosines, especially if small changes in DNA methylation are suspected - sequencing is not always accurate as one nucleotide (G or C depending on the sequenced strand) is heavily underrepresented in the sequence and matrices used by the sequencing software to decode original sequencing output are usually not adapted for spacing of nucleotide peaks in bisulfite-treated sequences, so one occasionally gets false positives.

As for converting the final alignment file into “black and white dots”, we do it in Excel. It’s not fancy but it works: delete all columns in the alignment in the Bioedit except columns where CpG dinucleotides should be. Then import this alignment into the Excel such that you have each CG column in one column. Then we simply replace CG with ● and TG with ○. Then adjust the cell and font size and text alignment within cells and paste the resulting table as a windows metafile into a Powerpoint presentation. For most purposes, this conversion is sufficient. For publication purposes we make the figure manually. Others ([comment 5](#)) use more sophisticated/higher throughput alternatives, such as the BiQ Analyzer program (Bock et al., 2005).

Materials & Reagents

LMP Agarose	(e.g. Invitrogen)
Lysis buffer	10 mM Tris-HCl, pH 7.6 10 mM EDTA 1% SDS
for 5 mL of Lysis buffer	50 μL of 1M tris-HCl, pH 7.6 100 μL of 0.5 M EDTA 250 μL of 20% SDS 4.6 mL of pure water

10 mM hydroquinone	We use SIGMA H9003-100G hydroquinone: 0.1101 g in 100 mL of H ₂ O or 0.055 g in 50 mL of H ₂ O
40.5% Sodium bisulfite	We typically use Acros Organics (NJ, USA) 419441000 Sodium bisulfite ACS. Sodium bisulfite and hydroquinone from Fisher also worked well. - dissolve 40.5 g (10.125 g) in ~ 80 mL (~ 20 mL) of H ₂ O, - adjust pH to 5.0 with 10M NaOH. Add water to 100 mL (25 mL)

Author Notes

- 20 ml of 2% [LMP Agarose](#) is more than sufficient.
- 2 mL tube allows for formation of nice beads which are not stuck to the bottom of the tube.
- Agarose should form a round-shaped bead in the oil. We use Eppendorf Thermomixer at 600 rpm to mix the sample and to facilitate bead formation.
- Eppendorf Thermomixer set at 400 rpm is very suitable for all incubations at higher temperatures.
- Use a rocking platform, rotating wheel, lambda grill, or a shaker to provide gentle shaking/mixing for all washes at room temperature.
- We always prepare 50 mL of hydroquinone and 25 mL of sodium bisulfite in 50 mL Falcon tubes for each bisulfite sequencing experiment. The reason for preparing 25mL is economical and practical – 25mL is the smallest volume, in which we could conveniently adjust pH of the bisulfite solution. We use SIGMA H9003-100G hydroquinone and Acros Organics (NJ, USA) 419441000 Sodium bisulfite ACS. Sodium bisulfite and hydroquinone from Fisher also worked well.
- We usually incubate for at least five hours.
- Converted material from very small samples should be used immediately. From my experience, when converting 200 ng of genomic DNA, once diluted, melted beads can be stored at -20 °C for months and even years.

Reviewer Comments

Reviewed by: Jörn E. Walter, Saarland University, Postbox 151150, 66041 Saarbrücken, Germany,
j.walter@mx.uni-saarland.de

- We also select primers manually but have good experience with lower Tms and slightly shorter Oligos (25-28bp).
- Hot start PCRs are really recommended. HotStart or Hotfire Pol work as well.
- We prepare smaller beads (max 10 µl and use 3 µl for the 30 µl PCRs).
- We agree; see some other suggestions outlined in our protocol.
- BIQ analysis is faster and more convenient in our hands.

Figures

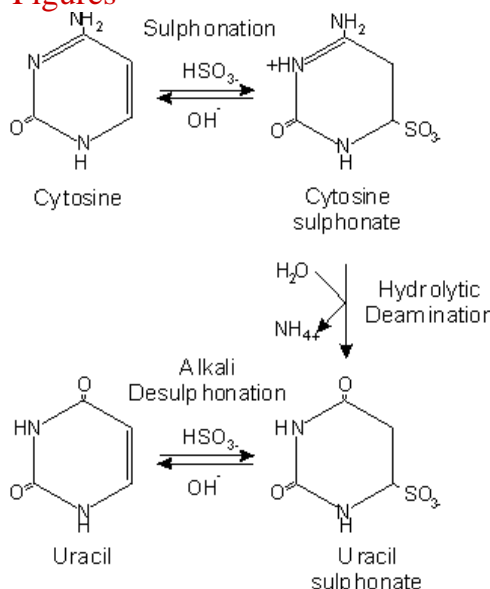


Figure 1. taken from: http://www.methods.info/Methods/DNA_methylation/Bisulphite_sequencing.html

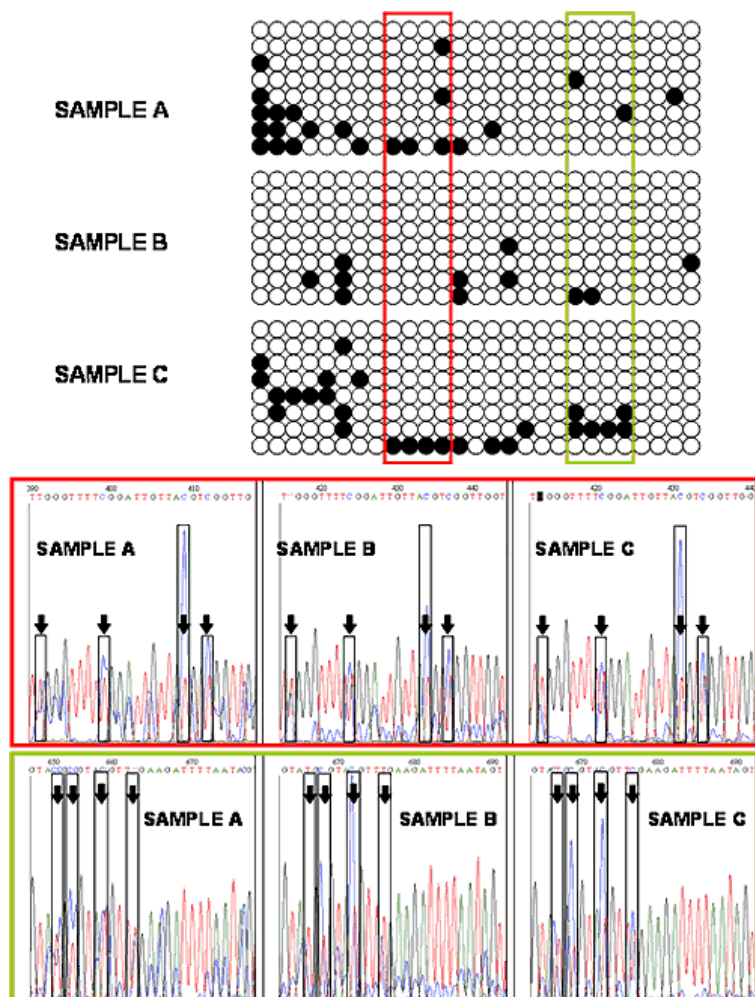


Figure 2. Direct PCR product sequencing may be misleading

Comparison of direct PCR product sequencing with results obtained after subcloning PCR products. Analyzed sequence is from a single-copy gene. Bisulfite sequencing was performed using 200ng of tail genomic DNA as a starting material.

On the top is shown analysis of individual clones from three different samples (8 clones per sample) suggesting low methylation within the analyzed region. Note that all clones containing at least one methylated cytosine are represented only once. This suggests that also clones carrying no methylated cytosines originate from independent DNA molecules in the sample.

The lower part shows sections of sequencing chromatograms corresponding to cytosines in colored frames above. Note that the signal from some unconverted cytosines (blue peaks) makes impression that these cytosines are highly methylated (e.g. position cytosine 3 in the red region).

This discrepancy is not due to inefficient cloning of methylated clones. Other experiments showed that methylated clones are cloned efficiently (data not shown). When in doubt, a suitable restriction digest can also provide a good insight into which fraction of PCR products carries CG or TG nucleotides in a specific position.

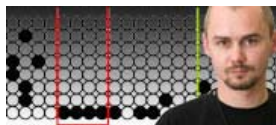
References

1. Bock, C., Reither, S., Mikeska, T., Paulsen, M., Walter, J. and Lengauer, T. 2005. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing *Bioinformatics* 21: 4067-4068.
2. Fedoriv, A.M., Stein, P., Svoboda, P., Schultz, R.M., and Bartolomei, M.S. 2004. Transgenic RNAi reveals essential function for CTCF in H19 gene imprinting. *Science (New York, NY)* 303(5655): 238-240.
3. Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences of the United States of America* 89(5): 1827-1831.
4. Olek, A., Oswald, J., and Walter, J. 1996. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic acids research* 24(24): 5064-5066.
5. Svoboda, P., Stein, P., Filipowicz, W., and Schultz, R.M. 2004. Lack of homologous sequence-specific DNA methylation in response to stable dsRNA expression in mouse oocytes. *Nucleic acids research* 32(12): 3601-3606.

Comments page 0 of 0:

[Add a Comment](#)

There are currently **0** comments to display.



© 2004-2009 Epigenome Network of Excellence
Printed from: <http://www.epigenome-noe.net/researchtools/protocol.php>