Protocol	#9.1
Title	**BOMB bisulfite conversion**
Keywords	DNA methylation, HT bisulfite conversion, Silica-beads
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Summary

Bisulfite sequencing [1] is considered the gold standard [2] for the analysis of DNA methylation status. Purified genomic DNA is treated with sodium metabisulfite which converts unmodified cytosines to uracils (which later amplify as thymines). Methylated cytosines (5mC) are resilient to bisulfite conversion and amplify as Cs. Thereby, the methylation status of each CpG site can be determined by sequencing and comparison to an unconverted reference sequence [3,4]. This is a modified, fast bisulfite conversion protocol and includes a rapid conversion, magnetic separation of treated DNA and on-bead desulfonation. This protocol was optimized for DNA amounts of 500-800 ng, but works well with input amounts ranging from 10-20 ng to 2 µg.

Chemicals

Name	Provider	PN	MW [g/mol]	Safety codes	
Ethanol (C_2H_6O, 99.9%)	Honeywell / Riedel-de Haën	34963	46.07	 Danger	H: 225-319 P: 210-80-305+351+338-308+313
Sodium metabisulfite ($Na_2S_2O_5$)	Sigma-Aldrich (Merck)	S9000-500G	190.11	 Danger	H: 302-318 P: 280-301+312+330-305+351+338+310
Hydroquinone ($C_6H_6O_2$)	Sigma-Aldrich (Merck)	H9003-100G	110.11	 Danger	H: 302-317-318-341-351-410 P: 201-273-280-305+351+338+310-308+313
N,N-Dimethylformamide (DMF, C_3H_7NO)	Acros Organics (Thermo Fisher Scientific)	210580025	73.09	 Danger	H: 226-312-319-332-360D P: 301+310-302+352-280-304+340-305+351+338-210
Guanidine hydrochloride ($CH_5N_3 \cdot HCl$)	Roth Chemicals	0037.1	95.53	 Warning	H: 302-315-319 P: 280-302+352-305+351+338
Sodium hydroxide (NaOH)	Roth Chemicals	6771.1	40.00	 Danger	H: 290-314 P: 280-301+330+331-305+351+338-310

Please consult appropriate MSDS information before working with these chemicals! Use lab coat, gloves and eye protection at all times! The chemicals are available from other providers as well. No preference is given to the indicated vendors.

Buffers and solutions

Silica-coated magnetic beads (20 µl per sample, 2 ml for a 96-well plate)

Take 1 ml of resuspended silica-coated magnetic beads (see **BOMB protocol #2.1**), pellet magnetically, and discard supernatant. Wash once with 1 ml of 7 M guanidine-HCl and resuspend in 2 ml 7 M guanidine-HCl

Conversion reagent (130 µl per sample, 13 ml for a 96-well plate, prepare freshly)

Reagent	Per 96-well plate
Sodium metabisulfite ($Na_2S_2O_5$)	6 g
Hydroquinone	5 mg
DMF	250 µl of stock
NaOH	3 ml of 2 M stock
ddH_2O	9.25 ml

Desulfonation solution (prepared fresh. 20 ml per plate)

0.25 M NaOH (2.5 ml of 2 M)
80% ethanol (16 ml of absolute, 1.5 ml water)

Binding buffer (if not dissolving, heat to 35 °C. 60 ml per plate)

7 M guanidine-HCl

90% ethanol (250 µl per sample, 25 ml per 96) – can be stored at RT for at least 12 months if closed properly

Elution buffer (60 µl per sample, 6 ml per 96) – can be stored at RT for at least 12 months

5 mM Tris-HCl pH 8.5

Equipment and setup

Fume hood

Heating block (e.g. Roth, Rotilabo®-block thermostat H250 – PN: Y264.1)

Thermocycler (e.g. BIOMETRA T1)

Microtiter-plate orbital shaker (e.g. IKA MS 3 basic)

Magnetic stand for 96-well plate (e.g. BOMB microplate magnetic rack)

Multichannel Pipettes

30 – 300 µl (e.g. Eppendorf, Eppendorf Research® plus 8-Channel – PN: 3125000052)

50 – 1200 µl (e.g. VWR, Multi-channel pipette, 8-channel – PN: 613-5422)

96-well PCR plate (e.g. Sarstedt – PN:72.1979.102)

1.2 ml 96-well deep well plates (e.g. Sarstedt, MegaBlock® 96 Well – PN: 82.1971.002)

Reservoirs (e.g. Roth, Rotilabo®-liquid reservoirs – PN: E830.2)

Seals (e.g. Bio-Rad, Microseal® 'B' Adhesive Seals – PN: MSB1001)

BOMB Bisulfite conversion

Step	Bisulfite conversion	Time	☑
1	Transfer 20 ng to 1 µg of your DNA sample in a total volume of 20 µl to fresh 0.2 ml PCR tubes or 96-well PCR plate		☐
2 ⚠	To each sample add 130 µl of **conversion reagent**, seal, mix well and spin down *DMF is highly toxic; sodium hydroxide and bisulfite are corrosive and dangerous!*	5 min	☐
3 ⚠	Place the samples in a thermocycler with a heated lid and execute the following program: Step 0 – 95 °C pause Step 1 – 95 °C 5 min Step 2 – 54 °C 30 min Step 3 – 95 °C 1 min Step 4 – 54 °C 30 min Step 5 – 95 °C 1 min Step 6 – 54 °C 30 min Step 7 – 4 °C storage for up to 20 hours *Converted DNA without further purification should not be stored for extended period of time due to progressing DNA degradation resulting in loss of DNA and decreased efficiency of the subsequent amplification step*	2 h	☐
Step	**Desulfonation and DNA purification**		
4	Pipette 20 µl of **silica-coated magnetic beads** (in 7 M guanidine-HCl) into the wells of a deep-well plate and add 600 µl of **binding buffer**	5 min	☐
5	Transfer the **converted sample** (150 µl from step 3) to the deep-well plate with beads in binding buffer	5 min	☐
6 ⚠	Shake for 5 min to mix and bind *If necessary, seal the plate and invert a few times to resuspend the beads*	5 min	☐
7	Pellet the beads with a magnetic rack and remove the supernatant. Wash twice with 400 µl of **90% ethanol**	10 min	☐☐
8	Desulfonate the DNA by adding 200 µl of **desulfonation buffer** and shaking at RT for **15 min**	20 min	☐
9	Pellet the beads with a magnetic rack, remove the supernatant and wash with 300 µl of **90% ethanol**	5 min	☐
10	Wash twice with 200 µl of **90% ethanol**	10 min	☐☐
11	Dry the beads by placing the plate at 50 °C for 10-30 min (until the beads become brittle and brown)	30 min	☐
12	Add 60 µl of **elution buffer** to the wells and shake shortly to resuspend (centrifuge shortly if beads stick to the walls)	5 min	☐
13	Pellet the beads magnetically and pipette off the eluted **bisulfite converted DNA** into a fresh plate or tubes	5 min	☐
End	Measure concentration	**~3.5 h** (1.5 h hands-on)	
	Store @ -80 °C (several weeks @ 4 °C is preferable to freeze-thaw cycles)		

Troubleshooting

Problem	Solution
Beads sticking to the sides	• Sonicate shortly in a sonic bath and/or push the beads down with a pipette tip
Beads stay at the bottom of the well when mixing	• Use a good foil to seal the plate and invert a few times to mix. Spin down briefly
Low conversion efficiency	• Ensure that you're using fresh conversion solutions
Low DNA amplification	• **DNA degradation:** Use more input DNA. If that is not possible, test skipping one cycle of 95 and 54 °C during conversion in the thermocycler • **Suboptimal primers:** use a set of validated PCR primers that are efficient in amplification of converted DNA

Exemplary Results

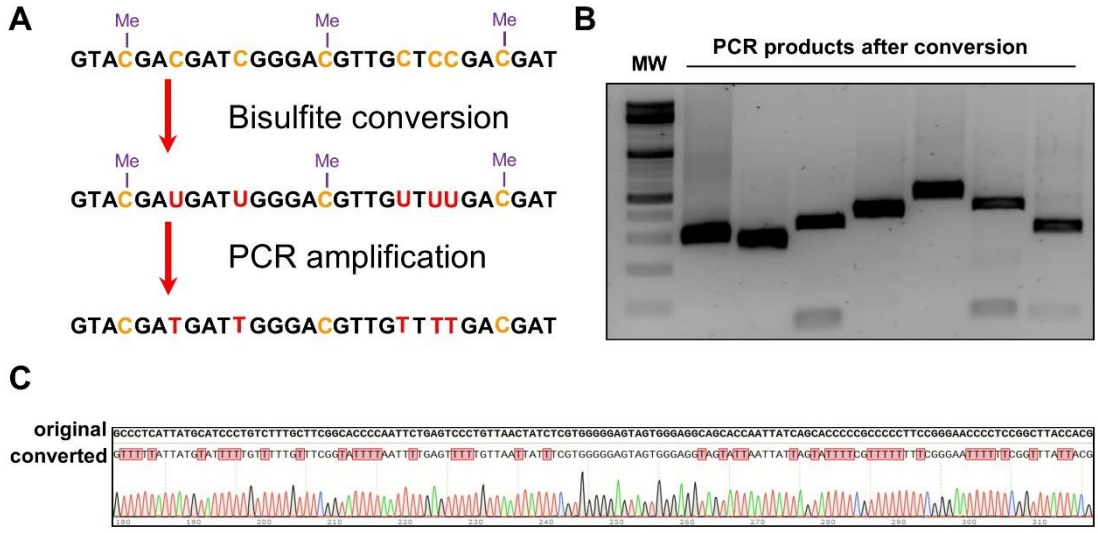

Figure S5: Bisulfite conversion. *(A) Scheme of DNA methylation analysis using bisulfite conversion. (B) Agarose gel after PCR amplification of bisulfite converted human DNA. Multiple primer pairs with expected product sizes between 221 and 435 bp were tested successfully. MW: GeneRuler DNA Ladder Mix, (Thermo Scientific). (C) Sequencing trace of a PCR amplified bisulfite converted sample aligned with the original, unconverted sequence. All non-CG cytosines were successfully converted. Conversion rate is ~99% as measured by sequencing after PCR amplification*

References

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