Jan 27, 2026

Assessment of DAPK1 and p16 promoter methylation by bisulfite conversion followed by MSP and COBRA in human tumour cell lines

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvo1xpdg4o/v1

Patricia Jiménez Cuadrado[1], Raquel Almansa[1], César García Martinez[1], Juan J. Tellería[1], Beatriz Merino[1], María Simarro[1], Miguel Ángel De La Fuente[1]

[1]Department of Cell Biology, Genetics, Histology and Pharmacology, Faculty of Medicine, University of Valladolid, 47005 Valladolid, Spain

Patricia Jiménez Cuadrado

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvo1xpdg4o/v1

Protocol Citation: Patricia Jiménez Cuadrado, Raquel Almansa, César García Martinez, Juan J. Tellería, Beatriz Merino, María Simarro, Miguel Ángel De La Fuente 2026. Assessment of DAPK1 and p16 promoter methylation by bisulfite conversion followed by MSP and COBRA in human tumour cell lines. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvo1xpdg4o/v1

Protocol status: Working
We use this protocol and it's working

Created: December 17, 2025

Last Modified: May 18, 2026

Protocol Integer ID: 235264

Keywords: promoter dna methylation in the p16, p16 promoter methylation, analysing promoter dna methylation, p16 promoter methylation by bisulfite conversion, absent promoter methylation, dapk1 gene, meaningful methylation pattern, methylation, cobra in human tumour cell line, different methylation state, assessment of dapk1, human tumour cell line, interpretable cobra restriction profile, unmethylated dna, specific pcr, combined bisulfite restriction analysis, bisulfite restriction analysis, human tumour, genomic dna, dapk1

Abstract

This protocol describes a three-session laboratory workflow for analysing promoter DNA methylation in the *p16 (CDKN2A)* and *DAPK1* genes using sodium bisulfite conversion followed by methylation-specific PCR (MSP) and combined bisulfite restriction analysis (COBRA). Genomic DNA from four human tumour-derived cell lines (HT29, HeLa, U2OS and 143B) is used to illustrate different methylation states.

Expected results include clear MSP amplification patterns specific for methylated and unmethylated DNA and interpretable COBRA restriction profiles, enabling the distinction between complete, partial and absent promoter methylation. The protocol supports structured interpretation of biologically meaningful methylation patterns as well as common sources of technical variability.

Guidelines

General considerations:
All procedures should be performed on a clean laboratory bench using standard laboratory practice. Laboratory personnel must wear appropriate personal protective equipment at all times, including gloves and a lab coat.

Sodium bisulfite is an irritant and should be handled with care. Preparation and handling of bisulfite solutions should be performed in a well-ventilated area, avoiding inhalation and contact with skin or eyes. Follow institutional chemical safety guidelines when handling and disposing of bisulfite-containing waste.

Genomic DNA used in this protocol is derived from established human tumour cell lines and should be handled as biosafety level 1 (BSL-1) material when purified DNA is used. No live cells or infectious material are involved.

DNA visualization should be performed using blue light transillumination whenever possible to minimize exposure to ultraviolet radiation. Appropriate protective eyewear must be worn during gel visualization.

All chemical and biological waste must be disposed of according to institutional biosafety and waste management regulations. Reagents and samples should be stored at recommended temperatures to ensure stability and reproducibility. Equivalent reagents or equipment may be used; however, the specified products are recommended to ensure consistency with the described workflow.

Materials

List of equipment

	A	B	C
	Item	Brand	Reference
	Elite Adjustable Volume Micropipettes (2 to 20 µl)	Fisherbrand	11845762
	Elite Adjustable Volume Micropipettes (20 to 100 µl)	Fisherbrand	11875762
	Elite Adjustable Volume Micropipettes (100 to 1000 µl)	Fisherbrand	11885762
	0.2 mL PCR tube rack (96-well, assorted colors)	Fisherbrand	HS23461AF
	Fluorescent rack for 1.5 mL microtubes (80 positions)	Fisherbrand	HS29025GF
	Dual stainless steel spoon/spatula, 150 mm	Fisherbrand	11533462
	Microcentrifuge (accuSpin Micro 17)	Fisherbrand	75002460
	DNA/RNA/Protein Quantification Spectrophotometer	Maestrogen	MN-917
	SimpliAmp Thermocycler	Applied Biosystems	A24811
	Isotemp Digital Dry Baths/Block Heaters	Fisherbrand	88-860-021
	ZX3 Vortex Mixer	Fisherbrand	F202A0176FI
	Motorized pipettor	Fisherbrand	15840053

	A	B	C
	Dual LED Blue/White Light Transilluminator	Invitrogen	LB0100

List of consumables

	A	B	C
	Item	Brand	Reference
	1–200 µL pipette tips, transparent, non-sterile (960 pcs)	Fisherbrand SureOne	T113RN-FIS
	100–1250 µL pipette tips, transparent, non-sterile (960 pcs)	Fisherbrand SureOne	T112NXLR-FIS
	0.2 mL PCR tube strip (8 tubes) with cap strip	Fisherbrand	14-230-215
	1.5 mL microtubes, natural, graduated, snapcap	Fisherbrand	509-GRD-PFB
	1-10 ml Disposable serological pipette	Fisherbrand	114434003
	15 mL centrifuge tubes, conical bottom, high clarity	Corning	352097
	50 mL centrifuge tubes, conical bottom, high clarity	Corning	352098
	250 ml glass bottles with stopper	Fisherbrand	SPFS33145
	Polystyrene weighing dishes, 88.9×88.9×25.4 mm (pack of 500)	Fisherbrand	08732114
	Filter paper, 42×52 cm, 500 sheets	Filtros Anoia	RM13054252
	Round LDPE bottles with key, 10 L	Nalgene	2318-0020

List of Commercial Kits

	A	B	C
	Item	Brand	Reference
	EZ DNA Methylation Kit	Zymo Research	D5001
	FastDigest Bsh1236I (BstUI)	Thermo Scientific	ER0922
	DreamTaq™ Hot Start PCR Master Mix	Thermo Scientific	K9012

List of Chemicals and Solutions

	A	B	C
	50× TAE buffer (Tris-acetate-EDTA) (4L)	Fisher Bioreagents	BP13324
	Agarose, low EEO, molecular biology grade (500 g)	Fisher Bioreagents	BP160-500
	Absolute ethanol (500 mL)	Fisher Bioreagents	BP2818500
	6x TriTrack DNA loading dye	Thermo Scientific	R1161
	SYBR Safe DNA Gel Stain	Fisher Bioreagents	S33102
	Mass Ruler Low Range DNA ladder	Thermo Scientific	SM0383

Troubleshooting

Problem
Low or absent PCR amplification
Solution
Verify DNA quality, bisulfite conversion efficiency, primer design, and PCR cycling conditions.

Problem
Incomplete COBRA digestion
Solution
Confirm enzyme activity, buffer compatibility, incubation time, and DNA input.

Problem
Non-specific bands or smearing
Solution
Reduce template amount, optimize annealing temperature, or decrease cycle number; verify primer specificity.

Problem
Unexpected digestion pattern in COBRA assay
Solution
Verify complete bisulfite conversion, confirm restriction site presence in the amplicon, and include methylated and unmethylated control DNA.

Safety warnings

Review the Guidelines & Warnings section carefully before performing this protocol, with particular attention to bisulfite handling and DNA visualization.

Ethics statement

This protocol does not involve experiments with animals or human participants. All procedures are performed using commercially available or previously established human tumour-derived cell line DNA samples. No ethical approval is required.

Before start

1. Preparation of methylated and unmethylated DNA controls (instructor-only):

Fully methylated DNA control:
Genomic DNA is enzymatically methylated *in vitro* using CpG Methyltransferase (M.SssI) (Thermo Fisher Scientific, Cat. No. 11368851). Briefly, genomic DNA is incubated with M.SssI in the presence of S-adenosylmethionine, allowing methylation of all cytosines within CpG dinucleotides.

Fully unmethylated DNA control:
Unmethylated control DNA is generated by whole-genome amplification using phi29 DNA polymerase (Thermo Fisher Scientific, Cat. No. 10233130) and Exo-Resistant Random Primers (Thermo Fisher Scientific, Cat. No. 10334450). This process produces DNA lacking CpG methylation, as epigenetic marks are not transferred during *in vitro* synthesis.

Use of control DNA:
Both methylated and unmethylated DNA controls are used exclusively for prior validation of MSP and COBRA primer specificity and are not included in the student laboratory workflow.

2. Genomic DNA used in the protocol (instructor-provided)

Genomic DNA from established human tumour-derived cell lines was used for bisulfite conversion and downstream MSP and COBRA analyses: HT29 (ATCC HTB-38), HeLa (ATCC CCL-2), U2OS (ATCC HTB-96), and 143B (ATCC CRL-8303). Genomic DNA was provided to students prior to the laboratory sessions.

3. Primer sequences used in this protocol

The following oligonucleotide primers were used for methylation-specific PCR (MSP) and COBRA analyses after bisulfite conversion.

- MSP p16
- M p16 SEN: 5′- TTGTTTTCGGTTGGTGTTTTC -3′
- M p16 ATS: 5′-CTCTTTCTTCCTCCGATACTAACG -3′
- U p16 SEN: 5′- TTGTTTTTGGTTGGTGTTTTTG -3′
- U p16 ATS: 5′-CCTCTTTCTTCCTCCAATACTAACA -3′

-COBRA p16
- COBRA p16 SEN: 5′-GGTGGGGTTTTTATAATTAGGAAAG-3′
- COBRA p16 ATS: 5′-AAACTAAACTCCTCCCCACCTAC -3′

-MSP DAPK1
- M DAPK1 SEN: 5′-TCGATTAGGCGTTTTGTGTC -3′

- M DAPK1 ATS: 5'- AAACAATCTCTCTCCAACCTACG -3′
- U DAPK1 SEN: 5′- GGTTTGATTAGGTGTTTTGTGTTG-3′
- U DAPK1 ATS: 5′-AAAAACAATCTCTCTCCAACCTACA -3′

-COBRA DAPK1

- COBRA DAPK1 SEN: 5'-GTTAGTTTTTGTTTTTTTAGTTAGG -3′
- COBRA DAPK1 ATS: 5′-ATCCCCAAAACCACATTCCTAA -3′

SECTION 1: DNA quality control and bisulfite conversion

1 **DNA quantification**

1.1 Select dsDNA measurement mode on the spectrophotometer.

1.2 Clean pedestals with Milli-Q water before and after each measurement.

SECTION 1: DNA quality control and bisulfite conversion

1.3 Blank using 2 µL TE buffer.

1.4 Measure 2 µL of each DNA sample and record:
- o DNA concentration (typically 10–100 ng/µL)
- o An A260/280 ratio in the acceptable range (1.8–2.0) indicates adequate DNA purity.

2 **Bisulfite conversion:** Bisulfite conversion was performed according to the manufacturer's instructions using the EZ DNA Methylation Kit (Zymo Research).

2.1 In a PCR tube, combine:
- o 200 ng genomic DNA
- o 5 µL dilution buffer
- o Nuclease-free water to a final volume of 50 µL

2.2 Incubate at 42 °C for 15 min.

2.3 Add 100 µL conversion reagent and mix gently.

2.4 Incubate at 50 °C for 12–16 h. Include an hourly 95 °C, 1 min denaturation step to ensure DNA remains single-stranded for efficient conversion.

2.5 After completion, hold samples at 4 °C until cleanup.

SECTION 2: Bisulfite cleanup and PCR amplification

3 **Bisulfite cleanup (Zymo-Spin column)**

3.1 Add 400 µL binding buffer to a Zymo-Spin column placed in a 2 mL collection tube.

3.2 Load the sample onto the column and centrifuge at ~16,000 × g (≈13,000 rpm, depending on rotor) for 30s; discard flow-through.

3.3 Add 100 µL wash buffer, centrifuge 30 s; discard flow-through.

3.4 Add 200 µL desulfonation buffer, incubate 15 min at room temperature, centrifuge 30 s; discard flow-through.

3.5 Wash twice with 150 µL wash buffer, centrifuging 30 s each time.

3.6 Transfer column to a 1.5 mL tube, add 20 µL elution buffer directly to the membrane, incubate 1 min, centrifuge 30s

3.7 Store bisulfite-treated DNA at −20 °C or proceed immediately to PCR.

4 **PCR amplification (MSP and COBRA)**

4.1 For each sample, set up three PCR reactions (50 µL each):
- MSP-M (methylated-specific primers)
- MSP-U (unmethylated-specific primers)
- COBRA (non-discriminatory primers)

4.2 Per 50 µL reaction:
- 25 µL 2× Taq PCR Master Mix
- 2 µL primer mix (forward + reverse, 10 µM each)
- 3 µL bisulfite-treated DNA
- Nuclease-free water to 50 µL

4.3 Cycling conditions:
- 95 °C for 1 min

- 38 cycles of:
  - 95 °C for 15 s
  - 57 °C for 15 s
  - 72 °C for 40 s
- 72 °C for 5 min
- Hold at 4 °C

Note

While the PCR is running,use SnapGene to visualize the p16 and DAPK1 sequences. Students must
locate the primers relative to CpG islands and discuss the expected amplicons based on the
chemical changes introduced by bisulfite conversion.
In addition, the instructor demonstrates how to use the MethPrimer web tool to identify CpG islands and to reproduce the design of the MSP and
COBRA primers employed in the practical session,
so that students understand the criteria used to select primer sequences for methylated, unmethylated, and non-discriminatory assays.

SECTION 3: COBRA Digestion and Gel Electrophoresis

5 **Restriction digestion (COBRA) :** Prepare two tubes per COBRA PCR product:

5.1 Digested sample (COBRA-D):
- 25 µL COBRA PCR product
- 3 µL 10× restriction buffer
- 2 µL Bsh1236I
- Final volume: 30 µL
- Incubate at 37 °C for 20 min

5.2 Undigested control (COBRA-ND):
- 25 µL COBRA PCR product
- 3 µL 10× restriction buffer
- 2 µL nuclease-free water
- Final volume: 30 µL
- Incubate at 37 °C for 20 min

6 **Agarose gel preparation**

6.1 Prepare a 2% agarose gel (1 g agarose in 50 mL 1× TAE buffer).

6.2 Heat until fully dissolved and allow to cool slightly.

6.3 Add SYBR Safe according to manufacturer's recommendations, pour gel, and allow to solidify.

7 Sample loading electrophoresis and visualization

7.1 MSP samples: mix 30 µL PCR product with 6 µL 6× loading dye

7.2 COBRA-D and COBRA-ND samples: add 6 µL 6× loading dye to each.

7.3 Load 5 µL DNA ladder (loading dye included)

7.4 Run gel at 140 V for approximately 15 min. Visualize bands using a blue-light transilluminator and document results.

7.5 Interpretation and expected student outcomes

At the end of the practical session, students should be able to interpret MSP and COBRA results to determine promoter methylation status across different tumour-derived cell lines. Students are encouraged to compare MSP and COBRA results across cell lines and discuss biological versus technical sources of variability.

Protocol references

Frommer M, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNAstrands. Proc Natl Acad Sci U S A. 1992;89(5):1827–1831.

Herman JG, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A.1996;93(18):9821–9826.

Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 1997;25(12):2532–2534.

Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002;18(11):1427–1431.

Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002;16(1):6–21.

Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128(4):683–692.

Acknowledgements

The authors would like to thank the Department of Cell Biology, Genetics, Histology and Pharmacology at the University of Valladolid for providing laboratory facilities and technical support.