

# Quantification of DNA methylation in electrofluidics chips (Bio-COBRA)

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Alterations of normal gene expression patterns are a hallmark of human cancers. It is now clear that the dysregulation of epigenetic modifications of the DNA and surrounding histones contributes to aberrant gene silencing, thus being major participants not only in the progression but also the initiation of the disease phenotype. The best-studied epigenetic modification is DNA methylation, which converts cytosine to 5-methylcytosine. Aberrant hypermethylation of the promoter is frequently observed in cancer and is generally associated with gene silencing. Currently, accurate and reproducible quantification of DNA methylation remains challenging. Here, we describe Bio-COBRA, a modified protocol for Combined Bisulfite Restriction Analysis (COBRA), that incorporates an electrophoresis step in microfluidics chips. Microfluidics technology involves the handling of small amounts of liquid in miniaturized systems. In the life sciences, microfluidics usually entails the scaling down of at least one application, such as electrophoresis, to chip format, which often results in increased efficiency and reliability. Bio-COBRA provides a platform for the rapid and quantitative assessment of DNA methylation patterns in large sample sets. Its sensitivity and reproducibility also makes it a tool for the analysis of DNA methylation in clinical samples. The Bio-COBRA assay can be performed on 12 samples in less than 1 h. If the protocol is started at the DNA isolation step, however, approximately 48 h would be required to complete the entire procedure.

## INTRODUCTION

The development and progression of human cancers is characterized by profound changes in cellular function, which stem from altered patterns of normal gene expression<sup>1,2</sup>. Over the past decade, it has become evident that genetic mechanisms account for only a subset of the alterations that typify the cancer genome<sup>3</sup>. It has also become apparent that epigenetic modifications of the genome, which are heritable modifications to the DNA without the alteration of the primary DNA sequence, are largely dysregulated in the cancer cell<sup>2,4-6</sup>. DNA methylation is the best-studied epigenetic modification, and it takes place predominantly in the context of 5'-CpG-3' dinucleotides<sup>7-9</sup>.

There are currently several approaches for evaluating DNA methylation. Most of these approaches, however, only assess DNA methylation in a qualitative manner<sup>10</sup>. Quantitative analysis of DNA methylation can be achieved using bisulfite DNA sequencing<sup>11</sup>. The main drawbacks of this technique are that it is costly and time consuming. Also, depending on the difference in the frequency of DNA methylation of the samples to be compared, a very large number of clones for each sample might have to be sequenced in order to attain statistically significant results.

We recently described the coupling of Combined Bisulfite Restriction Analysis (COBRA)<sup>12</sup>, a well-established method widely used in DNA-methylation laboratories, with an electrophoresis step in microfluidics chips (Agilent 2100 Bioanalyzer, <http://www.agilent.com>) for a rapid, accurate and cost-efficient quantification of methylation patterns in DNA samples of any

origin<sup>13</sup>. The main strength of our assay is that the DNA methylation status of all DNA molecules in a PCR product is assessed for each sample, which circumvents the need for sequencing individual clones. Bio-COBRA results are calibrated to a standard curve in order to extract *true* DNA methylation percentages. Our method, however, can be performed without a standard curve, if the analysis desired is a relative comparison of DNA methylation levels in a sample set. It should be noted, however, that Bio-COBRA analysis is limited to DNA sequences that possess at least one restriction-enzyme site with at least one CpG dinucleotide in its recognition sequence. Our method also provides a platform for the screening of DNA methylation in genomic regions where aberrant DNA methylation is known to occur, especially in genomic areas suspected of having diagnostic or prognostic value. Furthermore, Bio-COBRA can be used as a discovery tool to detect novel aberrant DNA methylation sites. Conversely, it is important to note that only the methylation status of the CpG dinucleotide or dinucleotides within the recognition sequence of the restriction enzyme used in the assay are interrogated for their methylation status.

It should be noted that Agilent Technologies is currently producing the Agilent 5100 Automated Lab-on-a-Chip Platform. This instrument allows investigators to perform Bio-COBRA assays using 96- or 384-well formats, thus affording Bio-COBRA the high-throughput capacity needed for the simultaneous screen of large sample sets.

## MATERIALS

### REAGENTS

• Genomic DNA isolated from individual patients, tissue samples or cell lines, prepared as described in REAGENT SETUP

• Genomic DNA isolated from peripheral blood lymphocytes (PBLs), prepared as described in REAGENT SETUP

• *Sss*I methylase (20 U  $\mu$ l<sup>-1</sup>) (New England Biolabs, cat. no. M0226L)



- 20 mM S-adenosylmethionine (SAM) (New England Biolabs, cat. no. B9003S)
- QIAquick gel extraction kit (Qiagen, cat. no. 28706)
- Platinum *Taq* DNA polymerase (Invitrogen, cat. no. 10966-034)
- 10 mM dNTP mix (Invitrogen, cat. no. 10297-018)
- Sodium bisulfite (Fisher Scientific, cat. no. S654-500)
- Hydroquinone (Sigma, cat. no. H 9003) **! CAUTION** Toxic
- NaOH pellets (Fisher Scientific, cat. no. BP359-212)
- Sodium acetate (Fisher Scientific, cat. no. S93352)
- 10× PCR buffer: 166 mM ammonium sulfate, 670 mM Tris pH 8.8, 67 mM MgCl<sub>2</sub>, 100 mM 2-mercaptoethanol
- DNase- and RNase-free water (Invitrogen, cat. no. 10977-015)
- Oligonucleotide primers designed against the target sequence of interest (10 pmol) (see REAGENT SETUP)
- Restriction enzymes: for example, *Bst*UI (cat. no. R0518L), *Hpy*CH4 IV (cat. no. R0619L), *Hha*I (cat. no. R0139L) (New England Biolabs) **▲ CRITICAL** Each restriction enzyme must have a CpG dinucleotide in its recognition sequence suitable for the analysis of the sequence of interest.
- 29% acrylamide (wt/vol) mixed with 1% N,N'-methylene-bisacrylamide (wt/vol) solution **! CAUTION** Toxic; avoid contact with the skin. Use a respirator when handling acrylamide and/or bisacrylamide in powder form.
- Ethidium bromide, 0.1% wt/vol (Sigma, cat. no. E 8751) **! CAUTION** Carcinogen
- 10× Tris/borate/EDTA (TBE): 0.90 M Tris, 0.90 M boric acid, 10 mM EDTA
- DNA 500 LabChip kit (Agilent Technologies, cat. no. 5064-8284)

**EQUIPMENT**

- GeneAmp PCR System 9700 (Applied Biosystems) or any thermal cycler

**PROCEDURE**

- 1| Fragment the genomic DNA using a sonicator at 70% power for at least 2 min. DNA fragments should not be larger than 2 kb.
- 2| To methylate genomic DNA *in vitro*, prepare the reaction mix as follows:

Fragmented genomic DNA	15 μg
<i>Sss</i> I	160 U
20 mM SAM	3.2 μl
10× enzyme buffer	40 μl
Water	to 400 μl

Incubate the reaction at 37 °C for 4 h. Two hours into the incubation, add an extra 2 μl of SAM and incubate for the remaining 2 h.

- 3| Recover the DNA from the reaction using QIAquick gel extraction kit columns. Mix the 400-μl reaction with 1,200 μl QG buffer (all buffers called for in this step are from kit) and 400 μl isopropanol. Vacuum the mix through the column. Wash twice with 800 μl PB buffer and spin the column at 16,100g for 2 min. Eluate the DNA 200 μl EB buffer.
- 4| Repeat Steps 2 and 3 once more.
  - ▲ CRITICAL STEP** Perform the *in vitro* methylation reaction on the same genomic DNA twice to ensure complete conversion of cytosine to 5-methylcytosine. DNA recovery from the columns can be maximized by performing the elution step four times, each with 50 μl elution buffer.
  - PAUSE POINT** The DNA can be stored for up to 6 months at -20 °C.

- 5| Prepare a 12-point DNA methylation standard as shown in **Table 1**. Adjust the concentration of the 100% *in vitro*-methylated DNA to 20 ng μl<sup>-1</sup>. Sonicate and adjust the concentration of PBL genomic DNA to 20 ng μl<sup>-1</sup>. Mix the 100% *in vitro*-methylated DNA with the PBL genomic DNA in ratios to obtain the following DNA methylation gradient: 0%, 1.6%, 3.2%, 6.4%, 12.5%, 25%, 50%, 75%, 87.5%, 93.6%, 96.8%, 100%.

**▲ CRITICAL STEP** The accuracy of the gradient rests first on the precise determination of the DNA concentration of both the 100% *in vitro*-methylated DNA and the sheared PBL DNA samples. Using a NanoDrop spectrophotometer can help obtain accurate optical density readings while minimizing sample loss. A second important consideration is that it is crucial to carefully pipette the indicated volumes so as to maintain the appropriate ratio of the two reagents.

- ND 1000 NanoDrop spectrophotometer (NanoDrop Technologies) or any spectrophotometer Agilent 2100 Bioanalyzer (Agilent Technologies)
- Concentrator 5301 (Eppendorf) or any vacuum dryer
- Sonicator
- Hybridization oven
- Water bath
- Electrophoresis power source
- Electrophoresis chamber
- Alphaimager UV transilluminator (Alpha Innotech) or any gel-imaging system

**REAGENT SETUP**

**DNA isolation** Freeze in liquid nitrogen ~100 mg human tissue with 1,000 μl lysis buffer (10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 100 mM NaCl, 0.5% wt/vol SDS). Crush the frozen tissue to a fine powder with a mortar and pestle. Add 10 μl proteinase K (10 mg ml<sup>-1</sup>) to the crushed tissue. Incubate the crushed tissue at 60 °C for at least 1 h. Extract the DNA using a mixture of phenol and chloroform. Dialyze the DNA sample against 4 liters 10 mM Tris, pH 8.0, to remove all traces of phenol. Precipitate the DNA using cold (-20 °C) 100% ethanol. As the genomic DNA samples isolated for Bio-COBRA are treated with bisulfite, the isolation of high-molecular-weight DNA is not required. Thus, commercially available kits, such as DNeasy (Qiagen) can be used to rapidly obtain low-molecular-weight DNA.

**Oligonucleotide primers** Design oligonucleotide primers to amplify the target sequence of interest. **▲ CRITICAL** To reduce the PCR bias toward the preferential amplification of either methylated or unmethylated sequences, primer binding sites should not contain CpG dinucleotides. Free online software to aid in the design of primers for DNA methylation analysis is available (<http://www.urogene.org/methprimer/index1.html><sup>14</sup> and <http://bisearch.enzim.hu>)<sup>15</sup>.

## PROTOCOL

**TABLE 1** | Example of a 12-point DNA methylation standard created by mixing 0% and 100% methylated DNA.

DNA methylation percent	0	1.6	3.2	6.4	12.5	25	50	75	87.5	93.6	96.8	100
<i>In vitro</i> treated DNA ( $\mu$ l)	—	0.8	1.6	3.2	6.25	12.5	25	37.5	43.75	46.8	48.4	50
Sonicated PBL DNA ( $\mu$ l)	50	49.2	48.4	46.8	43.75	37.5	25	12.5	6.25	3.2	1.6	—
Total volume ( $\mu$ l)	50	50	50	50	50	50	50	50	50	50	50	50
DNA amount ( $\mu$ g)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

### ? TROUBLESHOOTING

■ **PAUSE POINT** The DNA can be stored for up to 6 months at  $-20^{\circ}\text{C}$ .

**6|** Prepare a 3 M NaOH solution by dissolving 1.20 g of NaOH pellets in 10 ml of double-distilled water. To bisulfite convert and purify the DNA methylation standard, first add 5  $\mu$ l 3 M NaOH to each sample of the DNA methylation standard (50  $\mu$ l volume). Incubate the samples at  $42^{\circ}\text{C}$  for 30 min. After the incubation, add the following to each sample:

Denatured DNA standard sample	55 $\mu$ l
3 M sodium bisulfite, pH 5.0	515 $\mu$ l
10 mM hydroquinone	30 $\mu$ l
Final volume	600 $\mu$ l

Mix all reagents, wrap the samples in foil (to exclude light) and incubate at  $50^{\circ}\text{C}$  for 16 h in a hybridization oven.

**7|** Purify samples using a QIAquick gel extraction kit. To do so, add the following to each sample:

Bisulfite-treated sample	600 $\mu$ l
QG buffer	1,800 $\mu$ l
Isopropanol	600 $\mu$ l
Final volume	3,000 $\mu$ l

Mix all reagents well and vacuum through a spin column. Wash twice with 800  $\mu$ l PB buffer and spin the column at 16,100g for 2 min. Eluate the DNA 50  $\mu$ l EB buffer.

**8|** To complete the bisulfite treatment, add 5  $\mu$ l 3 M NaOH to each sample (50  $\mu$ l volume) and incubate at  $37^{\circ}\text{C}$  for 30 min. After the incubation, add 10  $\mu$ l 5 M sodium acetate to each sample.

**9|** Purify the samples using a QIAquick gel extraction kit by adding the following:

Bisulfite-treated sample	65 $\mu$ l
QG buffer	195 $\mu$ l
Isopropanol	65 $\mu$ l
Final volume	325 $\mu$ l

Vacuum the mix through the spin column. Wash twice with 800  $\mu$ l PB buffer and spin the column at 16,100g for 2 min. Eluate the DNA 300  $\mu$ l EB buffer.

▲ **CRITICAL STEP** All reagent solutions needed for bisulfite treating DNA (3 M sodium bisulfite, pH 5.0, and 10 mM hydroquinone) should be prepared fresh every time they are needed. Prevent their exposure to light by wrapping them in foil. Add 100–200  $\mu$ l 10 M NaOH per 10 ml 3 M sodium bisulfite solution to raise its pH to 5.

■ **PAUSE POINT** The bisulfite-treated DNA can be stored for up to 1 year at  $-20^{\circ}\text{C}$ .

**10|** Amplify by PCR the bisulfite-treated DNA methylation standard and samples of interest according to the reaction mix below:

Bisulfite-treated DNA	10 μl
10× PCR buffer	5 μl
10 mM dNTP mix	1 μl
Platinum <i>Taq</i> polymerase	1 U
Oligonucleotide mix (10 pmol each)	2 μl
DNase- and RNase-free water	up to 50 μl

Place the PCR reactions in a thermal cycler and activate the polymerase by incubating at 95 °C for 10 min. Carry out the amplification reaction for 35 cycles, using 96 °C as the denaturing temperature in each cycle: e.g., 95 °C for 10 min (96 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) × 35 cycles, 72 °C for 10 min.

**▲ CRITICAL STEP** Dimethylsulfoxide (DMSO) may be added to the PCR reactions to help amplify the target sequence. However, high DMSO concentrations (>5%) can inhibit the DNA polymerase. Thus, consider adding more units of enzyme if high DMSO concentrations are needed for your specific PCR reaction to work.

**? TROUBLESHOOTING**

**■ PAUSE POINT** The PCR products can be stored at 4 °C for up to 1 week.

**11|** Check the PCR amplification. Run 5 μl of each reaction in an 8% acrylamide gel. Run the gel for at least 1 h at 250V. Visualize the PCR products by staining the gel with a 0.001% ethidium bromide solution.

**12|** Clean the remaining PCR product (45 μl) using a QIAquick gel extraction kit by adding the following to each sample:

PCR product	45 μl
QG buffer	135 μl
Isopropanol	45 μl
Final volume	225 μl

Vacuum the mix through the spin column. Wash twice with 800 μl PB buffer and spin the column at 16,100g for 2 min. Eluate the DNA 50 μl EB buffer.

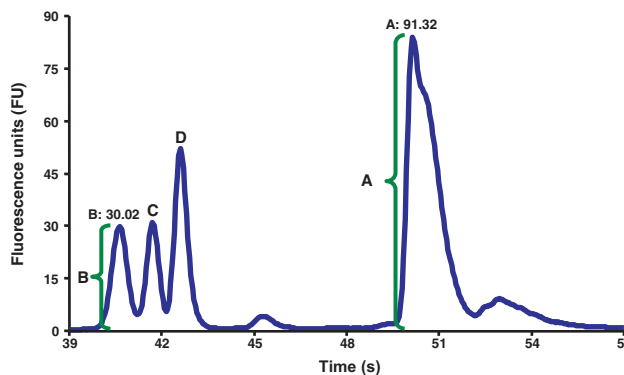
**■ PAUSE POINT** The purified PCR products can be stored at 4 °C for up to 1 week.

**13|** Concentrate the eluted PCR product to a final volume of 7 μl. Place the samples in a Concentrator 5301 heated to 60 °C. It takes approximately 15 min for the samples to reach the desired volume.

**▲ CRITICAL STEP** Concentrating the PCR samples is required in order to achieve a DNA concentration within the dynamic range of the Agilent DNA 500 LabChip chemistry (5–50 ng μl<sup>-1</sup>). This step is necessary if the expected restriction pattern (Step 10 and on) is made of multiple (four or more) restriction fragments.

**14|** Digest the PCR products with the appropriate restriction enzyme, which is selected based on the restriction sites present in the target sequence. Mix all reagents indicated below well and incubate at the appropriate temperature for at least 2 h.

PCR product	7 μl
Enzyme	10 U
10× buffer	1 μl
Water	up to 10 μl

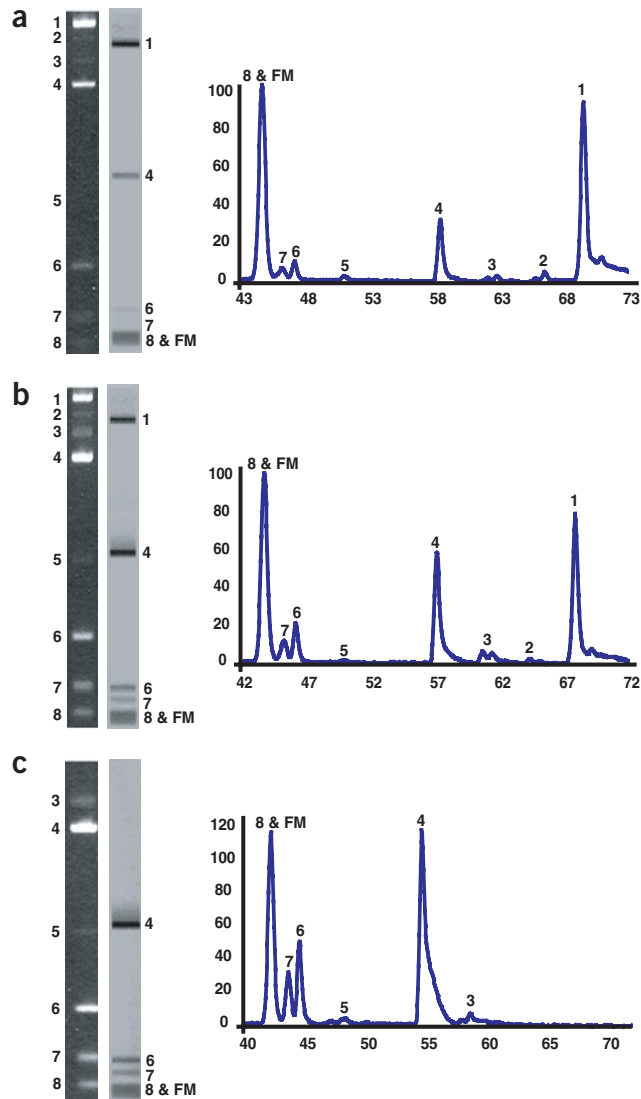


**Figure 1 |** Typical electropherogram generated by plotting Bioanalyzer raw data (CSV files) into Excel. The axis in the plot indicates fluorescence intensity (y) and migration time (x). Each peak in the electropherogram represents a DNA fragment. As in conventional DNA electrophoresis, DNA fragments in this system migrate according to their molecular weight. Because smaller fragments are detected first, their fluorescence intensities are recorded the earliest in the plot (39 s in the example provided). Peak A is the result of the signal generated by the undigested portion of the PCR product. Peaks B, C and D each represent a DNA fragment product of the restriction digestion carried out on the PCR product. The percent methylation of the sample shown is calculated by adding the fluorescence values of peaks B, C and D, divided by the addition of the fluorescence values of peaks A, B, C and D:  $[(B + C + D) / (A + B + C + D)]$ . The fluorescence units for each peak are calculated by measuring the height of each peak (examples A and B).

## PROTOCOL

**Figure 2** | Eight percent acrylamide gel, virtual gel and electropherogram visualization of a restriction-digested, 12.5% methylated sample.

(a) Restriction digestion of a 12.5% methylated sample separated by electrophoresis in an 8% acrylamide gel and subsequently stained with ethidium bromide (left lane). Virtual gel for the same sample automatically generated by the Bioanalyzer during the sample run (right lane) and the corresponding electropherogram (plot). It should be noted that low-intensity bands are visible in the acrylamide gel but are not present in the corresponding virtual gel. Nevertheless, all visible bands in the acrylamide gel can be matched to a peak in the electropherogram. The numbers to the left and the right of the gel lanes correspond to the peaks in the electropherogram. It should also be noted that in this example, a 17-bp fragment (indicated with the number 8) comigrates with the front marker (FM). The FM is a 15-bp DNA fragment included in all Bioanalyzer runs to calibrate the system. The overlapping of the 17-bp fragment with the front marker makes the 17-bp fragment unquantifiable. However, the elimination of this fragment from the quantification process would affect all samples equally, thus reducing the bias created by the elimination of one digestion product. Also, the low fluorescence intrinsically emitted from such a short fragment is, in most cases, only a small fraction of the total fluorescence generated by all the restriction fragments in the sample. The reason that the front marker peak measures 100 fluorescence units is because  $2.55 \times 10^{11}$  15-bp DNA molecules comprise that peak. (b) Same set up as in a, but of a 50% methylated sample. (c) Same set up as in a and b, but of a 96.5% methylated sample.



**PAUSE POINT** The digested PCR products can be stored at 4 °C for up to 1 week.

**15|** Check the restriction digestion. Run 5 µl of each reaction in an 8% acrylamide gel. Run the gel for at least 1.5 h at 250 V. Consider using a loading dye, such as YellowSub, that will not migrate over the expected restriction fragments. Visualize the restriction digestion by staining the gel with a 0.001% ethidium bromide solution. Check to ensure complete digestion was obtained in the 100% methylated sample, and that no restriction fragments are observed in the 0% sample (PBL control).

**CRITICAL STEP** Incomplete digestion in the 100% methylated sample may be attributed to either incomplete *in vitro* methylation or incomplete enzymatic digestion. Consider checking the *in vitro* methylation efficiency by PCR amplifying and digesting an aliquot of the *SssI* treated DNA before mixing the DNA methylation standard. Restriction digestion in the 0% sample could result from either basal DNA methylation in PBL DNA or incomplete bisulfite conversion.

### ? TROUBLESHOOTING

**16|** Follow exactly the protocol provided by Agilent for the DNA 500 LabChip. Load 1 µl of the restriction digestion into a DNA 500 LabChip well. Perform the electrophoresis step by running the Agilent 2100 Bioanalyzer

**CRITICAL STEP** Gel and dye must be equilibrated to room temperature before mixing and loading. Reset plunger of Chip Priming Station to 1 ml before opening station. This avoids spills to the seal, which could cause leakage of the station.

**17|** To maximize the efficiency of the assay, it is recommended to work with fully loaded chips (12 samples per run).

### ? TROUBLESHOOTING

**18|** Export the raw data (CSV file) for each of the samples and plot the raw data from each sample (fluorescence and migration time) in Microsoft Excel. The Agilent 2100 Bioanalyzer includes a software package that can be used to determine the peak area of each of the peaks generated during the electrophoretic run for each of the samples. However, we strongly encourage the use of peak height, instead of peak area, for calculating DNA methylation percentages<sup>10</sup>.

### ? TROUBLESHOOTING

**19** Measure the peak height for each of the restriction fragments and calculate the total DNA methylation level by using the following formula: fluorescence of methylated products/(fluorescence of methylated products + fluorescence of unmethylated product); see **Figure 1**. To help determine which peak corresponds to which restriction fragment, it is useful to generate a plot of the DNA size marker that is included in the chip with each experiment. The fragment size for each of the DNA markers is available in the Agilent DNA 500 LabChip chemistry product sheet. Truncated peaks, or lack of high-molecular-weight peaks, indicate the early titration of the fluorochrome used in the DNA 500 chemistry, probably as a result of DNA concentrations well above the dynamic range of the electrophoresis system (tested up to 65 ng  $\mu\text{l}^{-1}$ ). If these occur, consider performing the restriction reaction in a larger volume.

**? TROUBLESHOOTING**

**20** Compare the DNA methylation percentages obtained for each sample with the gel image generated in Step 17.

**21** Plot the 12 points of the DNA methylation standard in Microsoft Excel and generate a model. Use the equation from that model to derive the true methylation percentages for the experimental samples. Up to 12 Bio-COBRA samples can be processed in the 2100 Agilent Bioanalyzer in less than 1 h. However, if the procedure were to be started from the DNA isolation step, it would take approximately 2 d to complete the entire protocol.

**? TROUBLESHOOTING**

See **Table 2**.

**ANTICIPATED RESULTS**

Electrophoresis of digested PCR products generated from PCR reactions with average efficiency (~10–30 ng  $\mu\text{l}^{-1}$ ) yield fluorescence peaks well above background. The function derived from the 12 points in the DNA methylation standard is usually linear or logarithmic, depending on the difference of PCR amplification efficiency between the methylated and unmethylated

**TABLE 2** | Troubleshooting table.

PROBLEM	SOLUTION
Step 5 PBL DNA exhibits low-level methylation in the sequence of interest	One of the main advantages of Bio-COBRA is that DNA methylation percentages can be generated for each sample. If PBL DNA shows partial methylation at the sequence of interest, the analysis of a PBL sample will provide the baseline DNA methylation for that sequence. Thus, subtracting the background methylation level of PBL DNA from the unknown samples would yield the true increase in DNA methylation in those samples. Alternatively, DNA extracted from a different tissue in which no background methylation is present in the sequence of interest can be used instead of PBL DNA.
Step 11 Multiple bands in PCR amplification	Try using DMSO at various concentrations (1–5%) with concurrent changes in annealing temperature. It might also be useful to reduce the time in the extension step of the PCR cycle to 10–15 s.
Step 11 The PCR reaction generates primer dimers	Reduce the original amount of primer used per reaction by 50%. If primer dimers cannot be eliminated, accurate quantification of DNA methylation levels may still be possible. Calculate the size of all expected digestion products. If the primer dimer size does not overlap with any of the expected digestion fragments, the primer dimer fluorescence peak is simply ignored in the electropherogram analysis.
Step 16 Incomplete digestion of the 100% <i>in vitro</i> -methylated standard	Treat 100 ng SssI-treated DNA with bisulfite, amplify by PCR the CpG island of a housekeeping gene and directly sequence the PCR product. If C and T peaks overlap at any CpG dinucleotides, the <i>in vitro</i> methylation reaction was not complete. If only C peaks are detected at all CpG locations, the enzymatic digestion was not complete.
Step 17 Inefficient processing of samples in the Bioanalyzer	The DNA 500 Lab-on-a-Chip can accommodate 12 samples per run. Each chip can only be used once; thus, it is recommended that samples are processed, whenever possible, in multiples of 12 as a way to maximize the cost efficiency of the assay.
Step 18 Peak height versus peak areas to calculate DNA methylation percentages	For the purpose of the assay, accurate quantification of DNA methylation levels can be best achieved by measuring the peak height of each of the signals in the electropherogram <sup>13</sup> . To calculate the peak height of a signal, simply place the mouse arrow at the top of the peak in the electropherogram (graphed in Microsoft Excel).
Step 19 Interpreting the electropherogram generated by the Bioanalyzer	During separation of nucleic acids by electrophoresis in a DNA 500 Lab-on-a-Chip, DNA fragments migrate according to size. Thus small DNA fragments are detected before larger fragments. In the electropherogram, the <i>x</i> -axis represents time in seconds, increasing from left to right. Thus, peaks toward the left of the electropherogram are generated by the smaller DNA fragments that resulted from the restriction digestion. Conversely, larger fragments migrate further to the right.



molecules. A comparison between an 8% acrylamide gel, a virtual gel generated by the Bioanalyzer and the corresponding electropherogram is shown in **Figure 2**.

**COMPETING INTERESTS STATEMENT** The authors declare that they have no competing financial interests.

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