Sanger sequencing

Analyzing methylated DNA in liquid biopsies by Sanger sequencing

In this app note we:

- Introduce a workflow for analyzing methylated sequences in isolated DNA
- Demonstrate that differences in methylated sites can be analyzed in liquid biopsy samples
- Show that mixed methylation, either across the entire region or at specific sites, can be detected
- Provide a protocol for methyl DNA analysis starting from isolated liquid biopsy samples

Introduction

Biomarkers are biological characteristics that can be objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention (1). Examples include patterns of gene expression, levels of a particular protein in body fluids, or changes in electrical activity in the brain. In precision medicine, these biomarkers are often nucleic acid (DNA or RNA) based. Therefore, the importance and utilization of technologies that can analyze nucleic acid biomarkers is rapidly increasing. One subset of nucleic acid biomarkers that are becoming widely utilized are changes in DNA methylation patterns on specific sequences (for a recent review focused on cancer, see ref. 2). In eukaryotes, DNA methylation is usually found as 5-methylcytosine (5-meC) in CpG sites that are present in promoters and enhancers of genes. These methylated sites can affect the binding of DNA recognition proteins, and when DNA methylation status changes at these sites, it often results in changes in transcription of the associated gene.

Clinical researchers are increasingly collecting and analyzing biomarkers through liquid biopsies. These are non-invasive fluid collections, typically blood, in which circulating free DNA (cfDNA) or other biomarkers can be found (for a recent review, see ref 3). Because they are non-invasive, they are less stressful to collect samples for analysis, and facilitate longitudinal analysis of biomarkers.

DNA methylation offers many advantages over mutation detection when analyzing cfDNA when tumors are present (also known as ctDNA) (4). One of these advantages is that changes in methylation patterns can be associated with environmental insults that are not usually seen as DNA sequence changes. Hypermethylation can inactivate tumor suppressor gene expression, whereas hypomethylation can cause chromosomal instability and inappropriate expression of tumor growth genes (5). Methylation-based biomarker discovery and analysis using liquid biopsy samples is underway for many cancer types (6-8). Recently, using a next-generation discovery approach, Cristall et al described a panel for analyzing DNA methylation changes in triple-negative breast cancer using liquid biopsy samples (9).

Many different techniques are used to analyze methylation changes. One of the most common ways to discover changes is using next generation sequencing (NGS). While NGS excels at discovery-based approaches, the relatively high expense, involved workflows and data analysis complexity puts this out of reach for many researchers. On the other hand, PCR-based assays are relatively inexpensive and easy to perform, but only provide presence/absence information on the nucleotides being queried.

Sanger sequencing provides another attractive option for analyzing methylated DNA sequences. The workflow is relatively simple, fast and low cost. Data analysis of the peaks generated in an electropherogram is relatively straightforward; a reference genome based on bisulfite-modified DNA is not needed. Additionally, a single reaction can give information about all positions in the amplicon, facilitating the detection of partially modified sequences.

In this application note, we show how Sanger sequencing can be used to query specific methylated DNA sequences in liquid biopsy samples. We give some advice on designing primers and amplicons used for sequencing. Finally, we provide a protocol that can be used to analyze methylated DNA sequences by Sanger sequencing.

Workflow

A majority of the methylated DNA biomarkers of interest are in the form of 5-methylcytosine. Sequencing methods cannot detect the difference between 5-methylcytosine and normal cytosine – both pair equally well with guanine. However, unmethylated cytosine can be deaminated by treating with bisulfite, converting it to uracil that subsequently behaves as thymidine. Bisulfite conversion does not affect 5-methylcytosine, and it therefore continues to basepair as cytosine. Thus, after bisulfite conversion, any unmethylated cytosines change from CG base pairs to TA base pairs in subsequent assays, while any methylated cytosines maintain the CG basepairing. Bisulfite conversion completely changes a sequence, resulting in two new non-complementary strands, as illustrated in Figure 1.

Figure 1. Changes to DNA sequences produced by bisulfite conversion. A. Native dsDNA is annealed and complementary sequences are shown in red and green. Cytosines in 5'-CpG-3' sequences are methylated (yellow); all other cytosines are normal and unmethylated. **B.** Denaturation produces two single strands, each of which is modified by bisulfite treatment. **C.** Bisulfite treatment deaminates the normal cytosines and converts them to uracil. The methylated cytosines are protected, and therefore remain cytosines after treatment. Note that after bisulfite treatment, the two strands have different sequence and are no longer complementary. **D.** PCR is performed on the converted DNA using primers designed for the modified sequences (highlighted). Amplification converts the uracils to thymidines in the final sequence. Note that these PCR primers are different for the two starting strands, and these are usually different from the native genomic DNA sequence.

The example workflow for analyzing methylated DNA by Sanger sequencing is shown in figure 2. First, isolated DNA is treated with bisulfite to convert unmethylated Cs to Us. Next, the loci that will be analyzed are amplified using PCR primers that have been designed for the unique bisulfite-converted sequence. Note that DNA obtained from liquid biopsy (or FFPE samples) is fragmented, and the bisulfite conversion process fragments it further. Therefore, it is best to use primers that generate amplicons in the 80-120bp range. After PCR, the reaction is cleaned up by removing free primers using a solution like the ExoSAP-IT™ Express reagent. A portion of the PCR reaction is then used in the cycle sequencing reaction, followed by cleanup with a clean up kit such as BigDye XTerminator™ Purification Kit. Finally, the reactions are run on the CE instrument and peaks analyzed using standard software.

In the experiments described below, we used some of the sequences in the Cristall study (9). Among the genes we chose to analyze were four that illustrated methylation differences in our samples, as described below. To facilitate the Sanger sequencing, we modified the primers with M13 forward and reverse tags (Table 1). The M13 tags simplified setting up the cycle sequencing reactions by using a single mastermix for all loci. To isolate the liquid biopsy DNA samples, we purchased commercially-available breast cancer blood samples, and purified DNA from them using the King Fisher Flex automated purification system. We also used CpG uniformly-methylated human DNA, genomic DNA from MCF7 and T47D breast cancer cell lines, or commercially available CEPH genomic DNA. We converted the DNA samples using the EpiJet DNA conversion kit following the protocol provided. We then used the M13-tagged primers and Amplitaq Gold 360 mastermix to generate amplicons for sequencing. To help ensure that incorporated primers did not interfere with the sequencing reaction, we cleaned the PCR reaction with ExoSapIt. We then used the BigDye terminator 3.1 kit and M13 forward and reverse primers to cycle sequence the amplicons, and cleaned up the reactions with BigDye XTerminator. We sequenced the reactions on a SeqStudio Flex and 3500 Genetic analyzers. The results were analyzed using Genious Prime software. For details on the protocol, see the appendix of this app note.

Figure 2. Workflow for methyl DNA analysis using Sanger sequencing.

Table 1 Primers used in this study. Based off sequences from (9). Note that these primers contain CpG sites in the targeted region, and will therefore preferentially recognize methylated DNA when priming. However, unmethylated DNA between the primers can be detected, as illustrated.

Locus	Primer name	Primer sequence (M13 tags in red)	Location (hg18 coordinates)	Amplicon length
BRCA1	BRCA1-M13-f	GTAAAACGACGGCCAGTGGTAACGGAAAAGCGCGGGAATTATAGA	chr17:38530874-38530968	95
	BRCA1-M13-r	CAGGAAACAGCTATGACCCCCACAACCTATCCCCCGTCCAAAAA		
HOXB13	HOXB13-M13-f	GTAAAACGACGGCCAGTCGCGGGTTATAAATATTTGGTTGCGGC	chr17:44157793-44157885	93
	HOXB13-M13-r	CAGGAAACAGCTATGACCGCCACTACCTCGAAAACATTTCCC		
PDX1	PDX1-M13-f	GTAAAACGACGGCCAGGGAAAAAGGAGGAGGATAAGAAGCGCGG	chr13:27396588-27396685	98
	PDX1-M13-r	CAGGAAACAGCTATGACCCTCGCCGAAAATCACGACGCAATCCTAC		
OTXC	OTXC-M13-f	GTAAAACGACGGCCAGAGGGATTGTATTTCGAGGTGGTCGAGGT	chr14:56331673-56331781	109
	OTXC-M13-r	CAGGAAACAGCTATGACCCCGACAAATCGAAACCTTCGCCCGAAAC		

Results

The amount of DNA obtained from liquid biopsies can be low. The samples that we extracted yielded 10-20ng from about 1ml of collection. We used the maximal volume (20µl) for bisulfite conversion, and the final recovered yields ranged from about 1 to about 8 ng in 10µl, giving concentrations of 0.1-0.8 ng/µl. For the PCR amplification, we used 1 µl of converted DNA, following the protocol in the appendix.

In one set of experiments, we analyzed methylation status of a region of the BRCA1 gene (see Table 1). The primers for this region query five CpG sites. In the fully methylated genomic DNA sample, all of the cytosines in these sites (Figure 3A, red boxes) were protected, confirming that they were methylated. Similarly, genomic DNA from T47D cells also showed complete protection. However, genomic DNA from MCF7 cells showed small thymidine peaks under the cytosine peaks. In one position, this resulted in a mixed base call (indicated by Y). Similar results were seen when sequenced from the other direction (data not shown). Note that none of the other peaks are mixed, suggesting this locus is not uniformly methylated in MCF7 cells.

We next analyzed DNA isolated from liquid biopsy samples. In two of the samples (Figure 3B), these CpG sites showed a C➔T transition, indicating that were fully unmethylated. Note that this C➔T transition was seen when sequenced in either direction. For comparison, in the fully methylated sample these cytosines were fully protected, confirming that methylated cytosines could be distinguished. This region of BRCA1 contains binding sites for several transcription factors including E2F (10). These results suggest that BRCA1 tumor suppressor expression may not be affected by changes in transcription factor binding at this site.

In another set of experiments, we checked a region of the HOXB13 locus (Figure 4). This region also has five CpG sites that can potentially be methylated. We analyzed DNA isolated from two different FFPE-preserved breast cancer tumors. In one of the samples (Breast Tumor Sample 6), all of the cytosines in these sites were protected, indicating that they were methylated in the tumor. However, the other sample (Breast Tumor Sample 2), all of the cytosines in the CpG sites were heterogeneous, consisting of both Cs and Ts. This suggests that there was a mixture of methylated and unmethylated nucleotides in this sample.

Similarly, we analyzed the same region using liquid biopsy samples. For two of these samples, the CpG sites showed mixed base calls, suggesting that they too had mixed methylation status. These mixed bases could reflect one of two things in both the tumor sample and liquid biopsy samples: it could mean that the samples were heterozygous for either sequence or methylation state at the CpG sites, or more likely the mixed methylation could reflect tumor heterogeneity. Nevertheless, these results indicate that methylation differences can be detected in liquid biopsy samples.

Next, we examined results at the PDX1 locus. This region covers six potentially methylated CpG sites (Figure 5; only the site with differences is highlighted with the box). In the liquid biopsy sample 1, all of the CpGs were uniformly methylated. However, in the liquid biopsy sample 3, two of the CpG sites were mixed, while the other four appeared to be fully methylated. This suggests that differential distribution of methylated sites in a locus can be detected.

Finally, we analyzed results from the OTX2 locus (Figure 6). This region includes five CpG sites. In this experiment, we also included genomic DNA from CEPH genomic standard. For the CEPH DNA, we found that non-methylated cytosines make up four of the five sites; the last site appears to be fully methylated in this sample. In contrast, the DNA from liquid biopsy sample 3, MCF7 and CpG control DNA is fully methylated at all positions. Again, these results show that differentially methylated cytosines can be detected in a single locus.

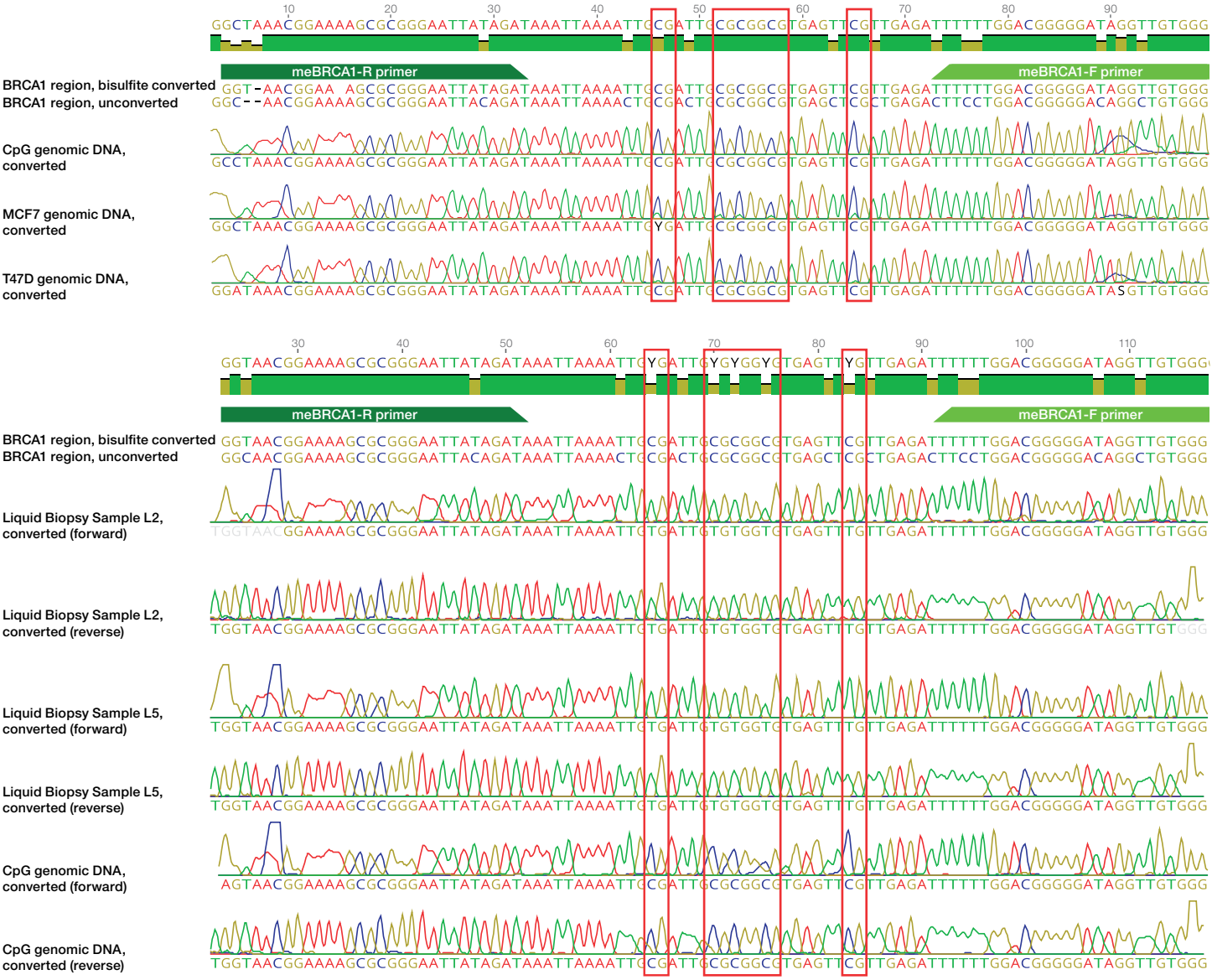

Figure 3. Analysis of methylation sites in BRCA1 promoter. In both panels, the predicted bisulfite converted and unconverted sequences are shown at the top, as well as the position of the primers used for PCR and cycle sequencing. Note that the position numbers are relative to the input sequence, and not the genomic coordinates. The electropherograms of each sample are shown below the reference sequences. **A.** Analysis in common breast cancer cell lines. In both cell lines, the CpGs highlighted by red box contained methylcytosine. In the MCF7 cells, the C at position 46 is partially methylated (indicated by a Y call). **B** Analysis in liquid biopsy samples In both L2 and L5 liquid biopsy samples, this region is unmethylated. The same sequence was observed when sequenced either direction. For comparison, the CpG fully methylated control DNA sample is shown.

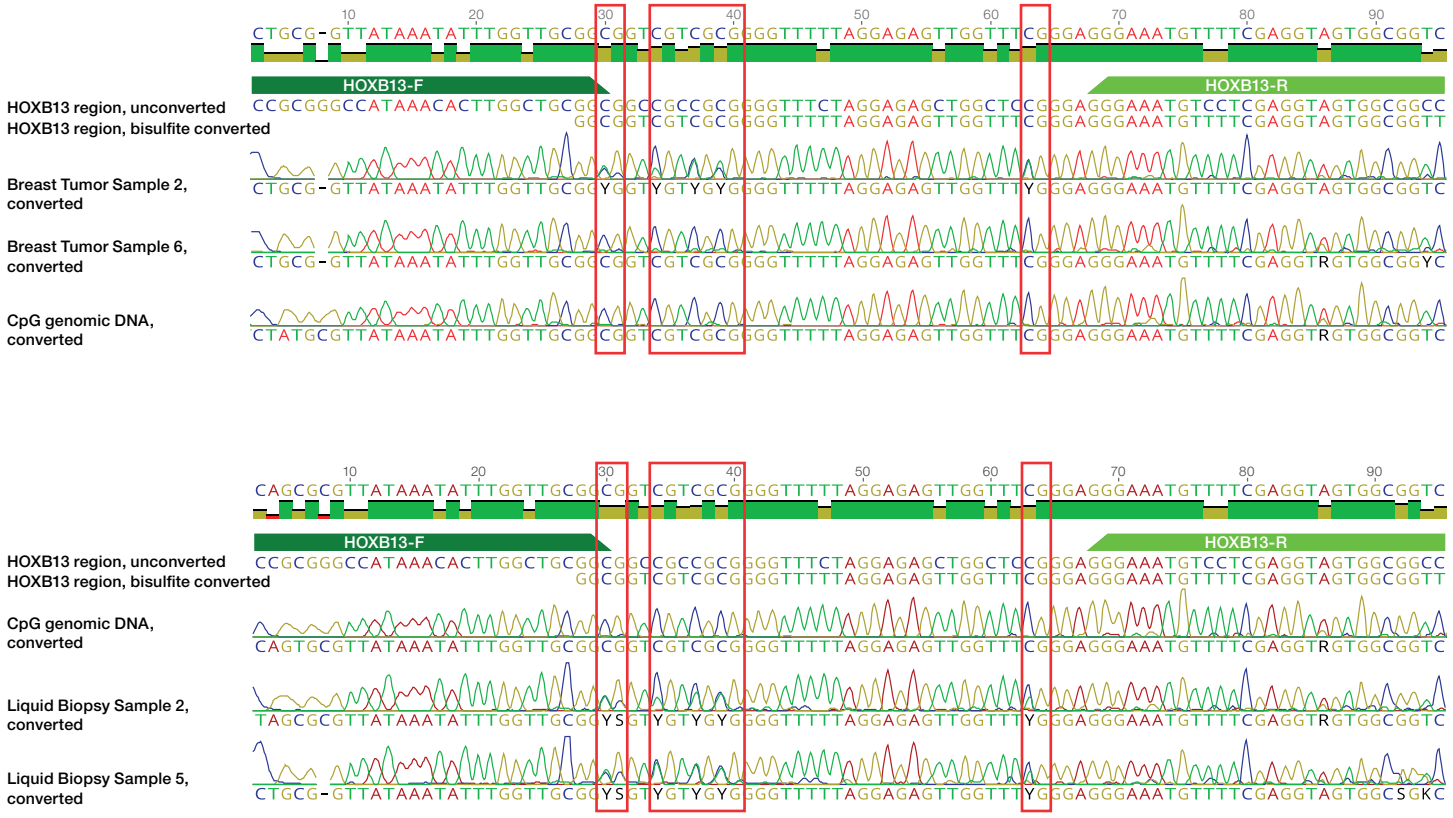

Figure 4. Analysis of methylation sites in HOXB13 locus. In both panels, the predicted bisulfite converted and unconverted sequences are shown, as well as the position of the primers used for PCR and cycle sequencing. Note that the position numbers are relative to the input sequence, and not the genomic coordinates. **A.** Analysis of two breast tumor FFPE samples. In Breast Tumor Sample 2, the CpGs are partially methylated, whereas the same locus in Breast Tumor Sample 6 is fully methylated. **B.** Analysis of two liquid biopsy samples. Both liquid biopsy samples show partial methylation at all CpGs in this region.

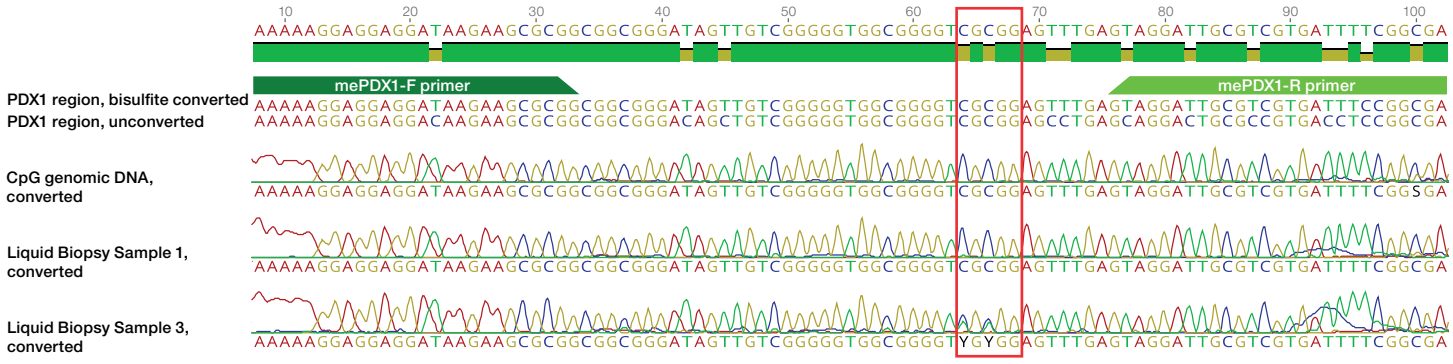

Figure 5. Analysis of methylation sites in PDX1 locus. The predicted bisulfite converted and unconverted sequences are shown at the top, as well as the position of the primers used for PCR and cycle sequencing. Note that the position numbers are relative to the input sequence, and not the genomic coordinates. In this region, the CpG fully methylated control and sample 1 are completely methylated, while sample 3 shows incomplete methylation at positions 65 and 67.

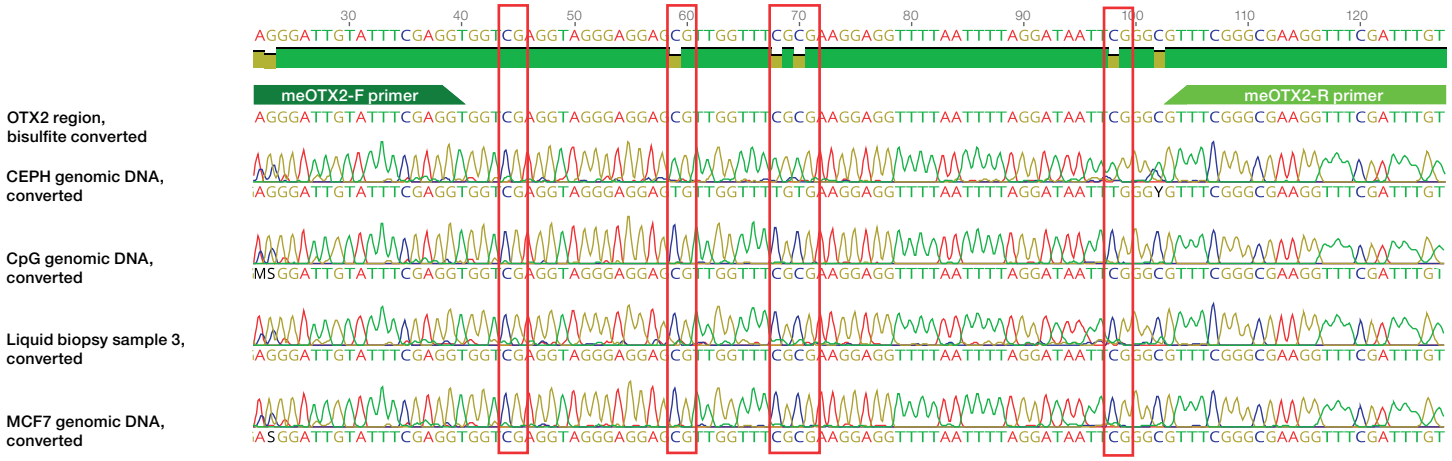

Figure 6. Analysis of methylation sites in OTX2 locus. In both panels, the bisulfite converted and unconverted sequences are shown, as well as the position of the primers used for PCR and cycle sequencing. Note that the position numbers are relative to the input sequence, and not the genomic coordinates. Here, the normal control CEPH genomic DNA is unmethylated at positions 59, 68, 70 and 98, but is fully methylated in CpG control DNA, and DNA from liquid biopsy sample 3 and MCF7 cells.

Summary

In this application note, we describe a method for analyzing DNA methylation in liquid biopsy samples using Sanger sequencing. We showed that by choosing small amplicons, the fragmented DNA in liquid biopsies can be effectively analyzed. We show that in addition to detecting fully methylated or unmethylated positions, we can also detect mixed methylation sites. These mixed sites could arise due to genomic heterozygosity, epigenetic heterozygosity, or tumor heterogeneity. We also show that different methylation states can be detected at different within a single region.

Finally, in the appendix, we provide a protocol that can be used to bisulfite convert input DNA, amplify specific loci, and sequence regions of interest to detect changes in methylation. Sanger sequencing has long been the go-to method for sequence determination and confirmation. It continues to be an important tool for research use due to its simple workflow, fast turnaround time and relatively low cost. The unambiguous nature of the data generated – the peak is the peak – can give investigators confidence in the results generated. For this reason, Sanger sequencing can be used to confirm DNA methylation changes obtained by discovery-based experiments, to focus on DNA methylation changes in specific regions using an efficient workflow and use liquid biopsies to analyze methyl DNA biomarkers.

Appendix

Protocol

1. Bisulfite conversion using EpiJET Bisulfite Conversion Kit

1.1. Preparation of Modification Reagent

1.1.1. Modification Reagent is supplied as a dry mixture and needs to be dissolved before use. The amount of Modification Reagent in each vial provided is sufficient for 10 DNA conversion reactions. Prepare only as many tubes as needed for current experiment.

1.1.1.1. For 10 conversion reactions:

- Add 0.9 mL of molecular biology grade water, 200 µL of Modification Solution I and 60 µL of Modification Solution II to a single vial of dry Modification Reagent.
- Dissolve Modification Reagent by inverting for about 10 min (trace amounts of undissolved bisulfite may remain).

Note: For best results, the prepared reagent should be used immediately. Modification Reagent solution can be stored for up to one week at 4ºC or 4 weeks in the dark at -20ºC. Modification Reagent solution taken from the refrigerator must be warmed to room temperature and vortexed prior to use.

1.2. Preparation of Wash Buffer

1.2.1. Add 25 mL of 96-100% ethanol to the 9 mL of Wash Buffer bottle before use. After the ethanol has been added, mark the check box on the bottle's cap to indicate that the step was completed.

1.3. Preparation of Desulfonation Buffer

1.3.1. Add 10 mL of 96-100% ethanol to the 3.5 mL of Desulfonation Buffer bottle before use. After the ethanol has been added, mark the check box on the bottle's cap to indicate that the step was completed.

2. Procedure

2.1. Add 20 µL of DNA sample containing 200-500 ng of purified genomic DNA into a PCR tube. If a volume of DNA sample is less than 20 µL, add water, molecular biology grade, to DNA sample up to 20 µL.

Note: Starting DNA amounts from 50 pg up to 2 µg can be used for DNA conversion using this kit. Nevertheless, for optimal results, use 200 ng - 500 ng of input DNA. High input DNA amounts may result in incomplete bisulfite conversion for some GC-rich regions.

2.2. Add 120 µL of prepared Modification Reagent solution to 20 µL of DNA sample in a PCR tube. Mix the sample by pipetting up and down, then centrifuge the liquid to the bottom of the tube.

2.3. Place PCR tubes into a thermal cycler and proceed with Protocol A to perform denaturation and bisulfate conversion of DNA:

Protocol A:

1) 98°C 10 minutes
2) 60°C 150 minutes
3) Proceed immediately to step 1.4. Alternatively, converted DNA can be stored at 4 °C overnight.

2.4. Add 400 µL of Binding Buffer to a DNA Purification Micro Column placed into a collection tube.

2.5. Load the converted DNA sample (from step 3) into the Binding Buffer in the column, mix completely by pipetting up and down.

2.6. Centrifuge the micro column and the collection tube at 12,000 rpm for 30 seconds. Discard the flow-through. Place the micro column back into the same collection tube.

2.7. Add 200 µL of the Wash Buffer, prepared with ethanol, to the micro column and centrifuge at 12,000 rpm for 30 seconds. Discard the flow-through. Place the micro column into the same collection tube.

2.8. Add 200 µL of the Desulfonation Buffer, prepared with ethanol, to the micro column. Mix well by pipetting up and down. Let the column stand at room temperature for 20 minutes.

2.9. Centrifuge the micro column placed into the collection tube at 12,000 rpm for 30 seconds.

2.10. Discard the flow-through. Place the micro column into the same collection tube.

2.11. Add 200 µL of Wash Buffer, prepared with ethanol, to the micro column and centrifuge at 12,000 rpm for 30 seconds. Discard the flow-through. Place a micro column into the same collection tube.

2.12. Repeat with a second 200 µL of Wash Buffer, prepared with ethanol, to the micro column and centrifuge at 12,000 rpm for 60 seconds.

2.13. Place the column into a clean 1.5 ml microcentrifuge tube (not provided). Add 10 µL of Elution Buffer to the micro column. Centrifuge at 12,000 rpm for 60 seconds.

Note: More than 10 µL (up to 20 µL) of Elution Buffer can also be used for converted DNA elution. If a small amount (< 1 ng) of DNA was used for DNA conversion, elute DNA with 6-8 µL of Elution Buffer. Repeat the elution step twice (for a total volume of 12-16 µL).

2.14. Eluted converted DNA is ready for downstream analysis. DNA can be stored at -20°C for more than 1 year.

3. PCR amplification

3.1. Prepare PCR master mix. Prepare the appropriate amount of master mix according to the table below:

Reagent	Amount per Reaction	Total amount for 25 reactions (example)
AmpliTaq Gold 360 PCR Master Mix	10 µl	250 µl
Locus-specific PCR primers (8nM concentration)	1 µL (final 400pM)	25 µl
Water	8 µL	200 µl
Total	**19 µl**	**475 µl**

Note: The amount of water will depend on the amount of sample added. These volumes are for 1 µl sample; adjust the volume of water so the total of sample plus water is 9µl if using more or less sample.

3.2. Aliquot 19 µl master mix into wells of a 96-well PCR plate

3.3. Add 1 µl bisulfite converted DNA (from step 2.14 above) to each well.

3.4. Seal the plate, vortex to mix, and spin plate at 1000g to collect everything to bottom of well

3.5. Run the plate using the following PCR profile:

Step	Temperature	Time	Number of Cycles
Polymerase activation	94°C	10 min	1
Amplification	95°	15 sec	40
	54-58°C (see note)	15 sec-30 sec (see note)	
	72°C	30 sec	
Final Extension	72°C	10 min	1
Hold	4°C	Hold	1

Note: the primers used to amplify bisulfite converted DNA may have a higher percentage of As and Ts than normal DNA. The annealing temperature and time can be varied for optimal amplification.

3.6. Proceed to PCR clean-up, or store the plate at -20°C until ready to use.

4. PCR Clean-Up

4.1. Add 8 µl ExoSAP-IT reagent to each reaction in the plate.

4.2. Seal the plate, vortex to mix, and spin plate at 1000g to collect everything to bottom of well.

4.3. Run the plate using the following PCR profile:

Step	Temperature	Time	Number of Cycles
Incubation	37°C	30 min	1
Inactivation	80°C	10 min	1
Hold	4°C	Hold	1

4.4. Proceed to cycle sequencing, or store the plate at -20°C until ready to use.

5. Cycle sequencing

5.1. Prepare BigDye Terminator master mix. Prepare the appropriate amount of master mix according to the table below:

Reagent	Amount per Reaction	Total amount for 25 reactions (example)
BigDye Terminator v3.1 Readymix	5 µl	125 µl
M13 primer	1 µL	25 µl
Water	3 µL	75 µl
Total	**9 µl**	**225 µl**

Note: M13 primers should only be used if the primers have M13 tags. Otherwise, use a single locus-specific primer used for PCR amplification.

Note: Prepare separate reactions for forward and reverse directions, if desired.

5.2. Aliquot 9 µl of master mix into wells of a new 96-well plate.

5.3. Add 1 µl of ExoSapIt-treated amplification reaction from step 4.4 above, to wells with mastermix. Reseal and store the master PCR plate at -20°C.

5.4. Seal the sequencing plate, vortex to mix, and spin plate at 1000g to collect everything to bottom of well.

5.5. Run the plate with the following PCR profile:

Step	Temperature	Time	Number of Cycles
Polymerase activation	96°C	1 min	1
Amplification	96°C	10 sec	25-30
	60°C (see note)	15 sec	
	60°C	4 min	
Final Extension	72°C	7 min	1
Hold	4°C	Hold	1

Note: Because the sequences may have a higher percentage of As and Ts than normal DNA, the annealing temperature and time can be varied for optimal amplification.

Note: It is not advisable to add more than 1µl of the PCR reaction to the sequencing reaction. If sequencing signal is low, try repeating and increasing the number of cycles.

5.6. Proceed to sequencing clean-up or store the plate at -20°C until ready to use.

6. Sequencing Clean-Up with BigDye Xterminator purification kit™

6.1. Prepare a mix with SAM™ Solution and XTerminator Solution in an appropriately sized tube. Clean-up will require 45µl of SAM solution and 10µl of XTerminator beads per well.

6.1.1. Add the amount of SAM™ Solution needed to a tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to resuspend. Cool to room temperature before using.

6.1.2. Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds, until the solution is homogeneous.

6.1.3. Using a wide-bore pipette tip, aspirate the correct amount of XTerminator Solution from the middle of the bottle. **IMPORTANT!** Avoid pipetting from the top of the liquid

6.1.4. Mix the XTerminator solution into the SAM solution until homogeneous.

6.2. Using a wide-bore pipet tip, add 55 µL of SAM Solution/ XTerminator Solution mix directly to each well of the cycle sequencing plate.

IMPORTANT! Avoid pipetting from the top of the liquid. Collect from the middle of the tube. When aliquoting into the plate, re-vortex the slurry every 8-12 wells to homogenize the bead mixture.

6.3. Seal the plate with MicroAmp™ Clear Adhesive Film. Make sure the plate is sealed well.

6.4. Vortex the reaction plate at maximum speed for 40 minutes

6.5. In a swinging-bucket centrifuge, spin the plate at 1500 × g for 2 minutes.

6.6. Proceed immediately to electrophoresis

7. Running the reactions on a capillary electrophoresis Genetic Analyzer

7.1. Make sure the instrument has been calibrated with Z-dye sequencing standards.

7.2. If needed, set up a run module:

7.2.1. For the SeqStudio 4-capillary Genetic Analyzer:

- Choose ShortSeq_BDX run module
- Z_BigDye Terminator v.3.1.
- Then, load the plate in the instrument and start the run.

7.2.2. For the SeqStudio Flex Genetic Analyzer:

7.2.2.1. For example, create a BDxStdSeq50_POP7_short module

7.2.2.2. Copy and modify existing BDx template. Change the parameters on the copied module as described:

- Oven temp: 60°C
- Injection voltage: 1600V
- Injection time: 6sec
- Run voltage: 8500V
- Pre-run time: 180sec
- Pre-run voltage: 15000V

7.2.2.3. When setting up plate, choose Z-BigDye Terminator v3.1 the plate and the new module

7.2.3. For the 3500 Genetic Analyzer – if needed, create a new run module

7.2.3.1. Copy and modify an existing BDx template. Change the parameters on the copied module as described:

- Dye set: Z
- Oven temp: 60°C
- Injection voltage: 1600V
- Injection time: 6sec
- Run voltage: 8500V
- Pre-run time: 180sec
- Pre-run voltage: 15000V

8. Analysis

8.1. Using a trace analyzing software such as SeqScreener Gene Edit Confirmation App, Sequencing Analysis Software, Geneious, or something similar, determine whether the trace quality is good enough to make confident calls.

For example, some guidelines might be:

- Trace score greater than 30
- CRL greater than 70% of the amplicon length
- QV20+ greater than 70% of the amplicon length

Note: These are suggested guidelines and not definitive rules. Individual requirements may differ.

8.2. Sequences that are good quality can be used to compare the bisulfite-modified sequences with unmodified, native sequence.

8.2.1. It is often helpful to also compare to a sequence that is fully bisulfite converted. This sequence can be generated using Methyl Primer Express™.

8.2.2. Alignment software such as Variant Reporter™ Software or Geneious can help line up the sequences for easy visualization.

Ordering information

Product	Quantity	Cat. No.
Instruments		
SeqStudio Genetic Analyzer System with SmartStart orientation Includes: SeqStudio Genetic Analyzer, SeqStudio data collection software, and 1-day SmartStart training,		A35644
SeqStudio Genetic Analyzer System with SmartStart orientation plus 1-year extended warranty Includes all items from A35644 plus additional 1-year warranty		A35645
SeqStudio Genetic Analyzer System with SmartStart orientation plus 3-year extended warranty Includes all items from A35645 plus additional 3-year warranty		A35646
SeqStudio Cartridge v2	1,000 reactions	A41331
SeqStudio 8 Flex Genetic Analyzer	1 system	A53627
SeqStudio 24 Flex Genetic Analyzer	1 system	A53630
Reagents		
Big Dye Terminator v3.1 reaction mix	1000 reactions	4337456
Big Dye XTerminator Purification Kit	1000 reactions	4376487
ExoSapIt Express PCR cleanup kit	2000 reactions	75001.4X.1.ML
EpiJet Bisulfite conversion kit	50 reactions	K1461
KingFisher Flex Purification system	1 system	24074410

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