

Methylation assay and sequencing using the Advalytix AmpliCell platform

Simple, sensitive and affordable protocols on epigenetics.

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The Advalytix AmpliCell – cell culture & PCR system is based on the AmpliGrid 1 μL reaction slide. The 48 hydrophilic reaction sites of the AmpliCell are treated with fibronectin to create an optimal surface for attachment of adherent cells. The hydrophobic regions outside the reactions sites are covered with a special foil that is easily removed for downstream PCR analysis. Disposable chambers for cell culture medium are pre-mounted on the slide to allow optimal growth conditions for the cells of interest. This application report shows data on the methylation state of cells grown on the AmpliCell platform using a simple, sensitive and affordable workflow.

Methylation of CpG islands in the promoter region of different genes that are involved in development and cancer progression, is assumed to have an influence on the expression of these genes. Either a hypomethylation or a hypermethylation leads to an increase in gene expression or silencing effect. The methylation state of different cell types such as cancer cells, stem cells or primary cells such as neurons is often important to understanding the influence of the methylation of genes.

Introduction

HeLa cells (HPV18 positive cervical carcinoma epithelial cells) are grown on AmpliCell (figure 1). A methylation assay using the Qiagen EpiTect® Bisulfite Kit followed by sequencing is performed to analyze the methylation status of genes.

1 Figure 1: AmpliCell platform



In the current example, we deposit HeLa cells on the AmpliCell and incubate for 3 hours to allow attachment of the cells on the AmpliCell surface. Cells are washed, dried and a bisulfite reaction is performed using a conventional kit. A PCR with methylation specific primers followed by a sequencing reaction is done. On the basis of the sequencing data it is possible to analyze the percentage of methylated versus unmethylated events.

Protocol

Stain HeLa cells with Hoechst dye (Hoechst Bisbenzimid H 33342, Sigma) with a concentration of 1mg/mL (100x staining solution). Dilute the Hoechst 1:100 with the cell suspension and incubate for 5-10 min at room temperature. Deposit 4×10^3 HeLa cells on the AmpliCell chambers (each reaction site will contain approx. 1×10^3 cells) and incubate for 3 hours to allow attachment of the cells to the AmpliCell surface.

Afterwards, remove chamber and foil of the AmpliCell and wash the glass slide with 1x PBS (phosphate buffered saline, pH 7.4), 0.05x PBS and water. Prepare the Qiagen Bisulfite mix as described in table A:

A Table A: Bisulfite mix

Component	1 reaction
Nuclease free water	0.14 μL
Bisulfite Mix	0.61 μL
DNA Protect Buffer	0.25 μL
Total volume	1 μL

Pipette 1 μL Bisulfite mix on each reaction site of the AmpliCell and immediately cover with 5 μL of sealing solution. Transfer the AmpliCell slide to the AmpliSpeed slide cyclor and run the program according to table B:

B Table B: Bisulfite program

Temperature	Time
99°C	5 min
60°C	25 min
99°C	5 min
60°C	85 min
99°C	5 min
60°C	175 min

Add 0.5 μL of 0.1M NaOH to each reaction site by pipetting on top of the sealing solution. Due to the physical conditions, the aqueous solutions will merge immediately. Incubate for 20 minutes at room temperature. Afterwards remove the sealing solution by dipping the slides into 100% hexane (please follow the safety precautions by handling with organic solvents). Wash the slide for 3 min in water and air dry it afterwards at 37°C using the incubation function of the AmpliSpeed slide cyclor.

To perform the PCR, pipette 1 μL of a methylation specific primer (forward and reverse; each 0.2 μM) on each reaction site of the AmpliCell and let them air dry at 37°C. Prepare the amplification master mix using the Qiagen Multiplex PCR Kit according to table C:

C Table C: Amplification master mix

Component	1 reaction
2x Qiagen Multiplex PCR Master Mix	0.5 μL
5x Q-Solution	0.06 μL
AdvaGold, 0.1%	0.1 μL
Nuclease free water	0.34 μL
Total volume	1 μL

Pipette 1 μL amplification master mix on each reaction site of the AmpliCell and immediately cover with 5 μL of sealing solution. Transfer the AmpliCell slide to the AmpliSpeed slide cycler and run the program according to table D:

D Table D: Amplification program

Temperature	Time	Cycle
96°C	10 min	
96°C	75 sec	
56°C	75 sec	45 cycles
72°C	75 sec	
72°C	10 min	

Afterwards analyze the samples by polyacrylamide gel electrophoresis and following silver staining or proceed with the sequencing reaction. To perform a sequencing reaction, add 0.4 μL of ExoSAP-IT® (USB Affymetrix) onto each of the reaction sites by pipetting on top of the sealing solution. Incubate the AmpliCell slide on the AmpliSpeed slide cycler for 60 min at 37°C and 15 min at 80°C. Perform the sequencing reaction by adding 1 μL nuclease free water on each reaction site and pipette two times 1 μL (each per sequencing reaction) to a AmpliGrid AG480F with predried primer (1 μL methylation specific primer, forward and reverse, each 0.3 μM). Add 1 μL BigDye Mix 1.1 (Applied Biosystems) on each reaction site and cover immediately with 5 μL of sealing solution. Run the program described in table E:

E Table E: Sequencing reaction program

Temperature	Time	Cycle
95°C	2 min	
95°C	45 sec	
63°C	45 sec	30 cycles
60°C	4 min	

For loading the samples into the sequencer, add 5 μL of nuclease free water on top of each reaction site and repeat this step once and add the aqueous phase in the same 1.5 mL tube. Optionally put the samples on a Sephadex column (G-50) before sequencing.

Results

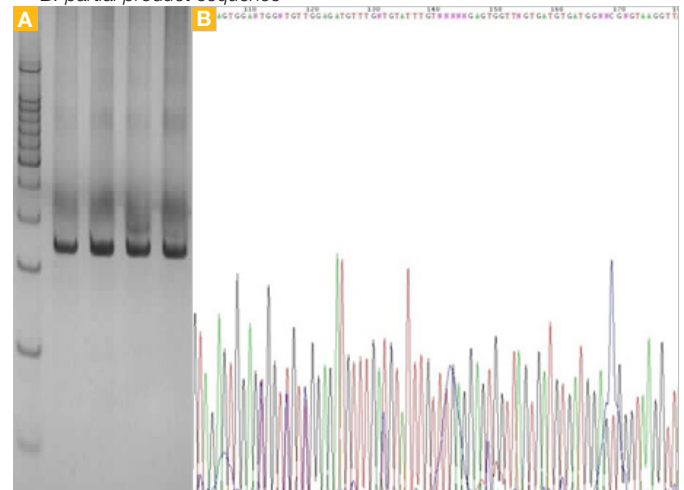
Bisulfite treatment of DNA selectively transforms all unmethylated cytosines into uraciles, whereas methylated cytosines are not affected by the reaction. AmpliCell is an excellent platform presenting a sufficiently high number of cells for methylation analysis. The methylation assay can be run directly on the cells without sample preparation. Sequencing of PCR products after bisulfite treatment shows the ratio of methylated and unmethylated cytosines.

Bisulfite treatment of HeLa cells cultured on AmpliCell can be done using a convenient assay without invasive detachment processes and loss of template material due to complex workflows. The presence of the 213bp band on the stained polyacrylamide gel shows the complete transformation of the DNA since a PCR product is only obtained from completely transformed DNA.

2 Figure 2: Analysis of products.

A: Gel analysis of products by PAGE (polyacrylamide gel electrophoresis; lane 5: 100 bp marker ;lane 2-5: specific band (213bp) from 1×10^3 HeLa cells)

B: partial product sequence



Discussion

The usage of AmpliCell for methylation assays and sequencing allows for analysis directly after cell cultivation without need of invasive treatments such as trypsination. No DNA extraction, column washing or other time consuming and error prone working steps are needed which makes AmpliCell the method of choice for simple and sensitive analysis of the methylation state of target genes.

Reagent and material saving makes the AmpliCell platform attractive for all methylation assays in epigenetics.

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