Chapter 3

Re-expressing epigenetically silenced genes by inducing DNA demethylation through targeting of Ten-Eleven Translocation 2 to any given genomic locus
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Re-expressing epigenetically silenced genes by inducing DNA demethylation through targeting of Ten-Eleven Translocation 2 to any given genomic locus

Chapter in Methods in Molecular Biology

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Abstract

Epigenetic editing is a novel methodology to modify the epigenetic landscape of any genomic location. As such, the approach might reprogram expression profiles, without altering the DNA sequence. Epigenetic alterations, including promoter hypermethylation, are associated with an increasing number of human diseases. To exploit this situation, epigenetic editing rises as a new alternative to specifically demethylate abnormally hypermethylated regions. Here, we describe a methodology to actively demethylate the hypermethylated ICAM-1 promoter. Reducing DNA methylation in our target region increased the expression of the ICAM-1 gene. As the ICAM-1 gene in our model cell lines was highly methylated (up to 80%), this approach proves a robust manner to reduce methylation for hypermethylated regions. Epigenetic editing therefore not only provides an approach to address mechanisms of gene expression regulation, but also adds to the therapeutic toolbox as current inhibitors of epigenetic enzymes are limited by genome-wide effects.

Keywords: Epigenetic editing, ICAM-1, TET2, DNA demethylation, zinc finger, pyrosequencing, transduction.

Introduction

Epigenetics can be defined as the study of stable changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence\(^1\). Epigenetic changes include chemical groups (marks) covalently attached to DNA and/or histones, changing their properties and altering their function. One of the most intensively studied epigenetic marks is the cytosine methylation (5mC), which along with other less common modifications\(^2\), occur generally in cytosines preceding a guanine (CpG). It consists of the covalent addition of a methyl group (CH3) to the position 5 of the pyrimidine ring of the cytosine. This reaction is mediated by a family of DNA methyltransferase proteins (DNMTs) which either maintain the methylation status on newly formed DNA chains (DNMT1) or methylate previously unmethylated positions (de novo DNMT3A and DNMT3B).

CpG sites for DNA methylation can be found either clustered in highly CG dense regions (called CpG islands (CGI)), scattered in less condensed CpG site regions (CpG shores) or dispersed along the genome as independent CpG sites. Depending on the genomic location, CpG methylation functions to repress gene transcription\(^3\): when located within promoter regions (transcription factor inaccessibility), close to known transcription start sites (TSS) of genes (diminishing expression of the mRNA or inducing alternative TSS usage), or inside the gene bodies (repressing the activity of intragenic promoters -such as those driving the expression of noncoding RNAs- and even related with splicing alterations)\(^4\)-\(^10\). DNA methylation regulates transcription by altering the molecular structure of the cytosines which impairs the interaction between DNA and their binding proteins, such as transcription factors, which often are sensible to this mark\(^11\). On the other hand, methylated CpG sites are recognized by methylated CpG binding proteins (like MBDs)\(^12\) which recruit other transcription repressors as histone deacetylases (HDACs), inducing chromatin structure modifications which will also influence the transcription factors and transcription machinery accessibility.
As all epigenetic marks, DNA methylation is a dynamic and reversible process, and DNA demethylation can occur as an active or passive event. Passive DNA demethylation occurs when a DNA methylation mark is not copied to the newly formed DNA strand after replication, this creates hemimethylated sites, which will be lost upon subsequent cell division. Active DNA demethylation, on the other hand, refers to the enzymatic process by which 5mC mark is oxidized in several steps to 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (5mC->5hmC->5fC->5caC). To recover the cytosine base in DNA chain, 5fC and 5caC can be excised by thymidine DNA glycosylase (TDG) leaving an abasic site which in turn is recognized and repaired with an unmodified cytosine by base excision repair (BER) system.

Human Ten-Eleven Translocation (TET) family enzymes, previously related to hematopoietic malignances, have been identified in 2009 as the responsible enzymes for 5mC->5hmC oxidation; its identification as possible mediator in 5mC->5hmC oxidation was initially identified due to their homology to the trypanosomal proteins JBP1 and JBP2 (members of the Fe(II)/α-ketoglutarate (α-KG)-dependent oxygenase family of enzymes) which mediate thymidine oxidation to 5-hydroxymethylurasil (5hmU) in this organism. Further functional experiments successfully validated TET family enzymes as responsible of 5mC oxidation in humans. At the time of identification of the TET enzymes, we were pioneering the concept of epigenetic editing using engineered Zinc Finger Proteins, through which a desired genomic region can be targeted and any epigenetic mark present nearby can subsequently be modified by the fused epigenetic enzyme. This rewriting of the local epigenetic signature can potentially be further optimized to obtain the desired stable effect on gene expression.

Targeting specific regions of any genomic location nowadays can be achieved through different methodologies; most of them taking advantage of DNA binding domains (DBDs) of naturally occurring proteins, including helix-turn-helix, Zinc-Fingers, leucine zippers, winged helix, helix-loop-helix, HMG-box, immunoglobulin fold, B3 domain, and more recently transcription activator like effectors (TALEs). These domains vary in size, length recognition capacity, affinity, and structure. Specific DNA sequence recognition can also be made using a small single stranded DNA sequence in what is called Triplex Forming Oligonucleotide (TFO), or by the combination of RNA-protein as the case of the CRISPR/CAS system.

Development of DNA targeting methodologies, especially the CRISPR-Cas revolution, currently allows us to regard the genome as a tunable structure, susceptible of editing. Most attention is being paid to specific induction of genome sequence changes by targeting DNA nucleases to a given genomic locus (genome editing). The introduced double strand break will either induce homologous recombination or is repaired by the error-prone non-homologous end joining; the last can be used to inactivate genes and this concept has been tested in various clinical trials using engineered ZFPs. Additionally, the possibility to modulate gene expression without affecting the genome sequence by using epigenetic proteins, fused to these DNA targeting tools, is now increasingly appreciated. These fusion proteins can modify the epigenetic landscape of any genomic region (epigenetic editing) in order to induce or prevent gene expression.

In both genetic and epigenetic editing, programmable DNA targeting strategies are always necessary to interact with a desired region of DNA. The most common DNA targeting platforms for epigenetic editing include Zinc Fingers, TALEs, and the CRISPR/dCas9 system (targeting a catalytically dead Cas9 protein). The first and most extensively studied programmable DBDs are the Zinc Finger proteins, which are modular proteins consisting of individual “fingers” able to recognize three nucleotides each. Each finger is composed of around 30 amino acids, stabilized by a zinc ion which frequently bind to two cysteine and two histidine residues (Cys2His2-type). Based on its modular character, fusion of six fingers together extends the recognition sequence size to 18 base pairs which is enough to recognize unique sites in the human genome. Vast information about zinc fingers DNA binding rules, resulted in the postulation of the “recognition code,” which allow the rational designing of zinc fingers targeting any sequence.

An increasing amount of human diseases including different kinds of cancer and syndromes, are now being related to epigenetic abnormalities such as aberrant histone modifications and hypo- or hyper- DNA methylation related with atypical expression of certain genes.
Specifically in cancer, transcriptional silencing by hypermethylation has been reported for key regulatory genes related with cell cycle or apoptosis; in this kind of situations, enforced DNA demethylation via targeting of such altered genes, using epigenetic editing, offers a novel approach for intervention and correction of specific gene expression abnormalities. Currently, DNA demethylation is clinically achieved with treatments as azacitidine (5-azacitidine) and decitabine (5-aza-2’-deoxicitidine), but these treatments function genome-wide and thus are limited in their clinical applications.

Several databases are available these days for a number of human and non-human cell lines and patient samples where gene expression is coupled to epigenetic marks. Such studies facilitate the choice of potential study models for epigenetic editing studies. In this regard, C13ORF18 was identified as frequently hypermethylated in cervical cancer specimens, but not in normal cervix scraping. We assigned tumor suppressive function to this gene by re-activating its expression, first by targeting the transcriptional activator VP64, next by re-expression via induced DNA demethylation. The concept was further validated for other tumor suppressor genes.

In our first report on inducing DNA demethylation, we compared TET1, TET2 and TET3 and concluded that TET3 was not effective and that TET2 was somewhat more effective than TET1. Our first model genes for targeted demethylation were the InterCellular Adhesion Molecule 1 (ICAM-1) and the Epithelial Cell Adhesion Molecule (EpCAM) gene promoter. The ICAM-1 promoter is known to be silenced by specific CGI-related hypermethylation and previous reports demonstrate it is susceptible for reactivation using zinc finger protein coupled to a transient activation domain (VP64). In this chapter, we present the protocol specially designed for lowering the methylation percentage of the hypermethylated ICAM-1 promoter in human ovarian carcinoma cell line A2780. Here we induce Zinc Finger-TET2 fusion protein expression using a retroviral GFP reporter model; transduced cells are sorted from untransduced cells by FACS and finally ICAM-1 expression and promoter methylation status is quantified by qRT-PCR and pyrosequencing, which allow confirmation of TET2 activity in the chosen model. Other DNA targeting platforms have further validated the potency of targeting TET1 to reduce hypermethylation status inducing gene expression. Altogether, the approach of targeted demethylation opens realistic avenues to start considering therapeutic re-expression of aberrantly silenced genes or of (fetal) genes which can compensate for a genetic mutation.

Materials

1. Transduction of cells to express ZF-TET2
   1. HEK293T and A2780 cell lines (human embryonic kidney and ovarian cancer cells, obtained from ATCC)
   2. HEK293T Cell culture media: 500 mL Dulbecco’s Modified Eagle Medium (DMEM), 50% fetal bovine serum (Perbio Hyclone), 5mL L-glutamine (200mM) (Bio Whittaker), 3 mL gentamycine (10mg/mL) (Bio Whittaker).
   3. Calcium Chloride (CaCl$_2$) 2.5M: 36.75 g CaCl$_2$·2H$_2$O (Merck) +100mL H$_2$O miliQ (mQ). Filter the solution using a 0.2 µm filter.
   4. Polybrene (Hexadimethrine bromide) solution 1mg/mL: 50 mg Polybrene (Sigma) + 50 mL H$_2$O mQ.
   5. PBS four salts (NaCl, KCl, Na$_2$HPO$_4$, KH$_2$PO$_4$).
   6. HBS 2x solution: 0.28 M NaCl, 50mM HEPES, 1.42 mM Na$_2$HPO$_4$, pH 7.06. Filter the solution using a 0.2 µm filter.
   7. VSV-G envelope expression plasmid (pMD2.G) and Gag-Pol expression plasmid (pMDLg/pRRE)34.
   9. 10 cm cell culture plates
   10. T75 flask
   11. 0.45 µm SFCA syringe filters
2. Sorting of GFP positive cells
   1. PBS four salts
   2. TEP (Trypsine-EDTA-PBS) solution: 478.5 mL PBS, 10 mL trypsin, 2.5 mL EDTA (10 mM)
   3. HEK293T cell culture media

3. DNA extraction
   1. 6 well culture plates
   2. Chloroform
   3. NaCl 6M solution
   4. Ice cold Isopropanol
   5. Ice cold Ethanol 75%
   6. EDTA 0.5 M, pH 8
   7. SDS 10%
   8. SE 10x Solution: 125 ml NaCl 6M, 500 mL EDTA 0.5 M pH 8, adjust volume to 1 L.
   9. TE buffer: 10 mM Tris and 0.1 mM EDTA pH 8.
   10. Proteinase K (Roche)
   11. RNase (Qiagen)

4. Pyrosequencing
   1. Agarose
   2. Tris-Acetate-EDTA (TAE) Buffer: Tris base 40mM, EDTA 2mM, acetic acid 20 mM
   3. Streptavidin Sepharose High performance (GE healthcare)
   4. PyroMark™ Q24 pyrosequencer (Qiagen).
   5. PyroMark™ Q24 vacuum work station (Qiagen).
   6. PyroMark™ Q24 plates (Qiagen).
   7. PyroMark™ PCR kit (Qiagen).
   8. PyroMark™ Wash Buffer (Qiagen).
   9. PyroMark™ Denaturation Buffer (Qiagen).
   10. PyroMark™ Annealing Buffer (Qiagen).
   11. PyroMark™ Binding Buffer (Qiagen).
   12. PyroMark™ Gold Q24 reagents (Qiagen).
   13. Primer Pyro-F (5´-GGGGAAGTTGGTAGTATTTAAAAGT-3´).
   15. Primer Pyro-seq ( 5´- TGGGGGAGGGGAGTTTATT-3´).

Method

1. Transduction of cells to express ZF-TET2
   Zinc Finger construction
   1. Determine the sequence to be targeted (see Note 1)
   2. ZF are constructed by building blocks, using previously described recognition modules as de-
      scribed by Barbas 42-44, Kim 45 or Young 46. Nowadays, it is possible to use bioinformatics tools
      to select target sequences and design the proper ZF (see Note 2).
3. Assemble the ZF blocks using canonical peptide TGEKP as linker. We suggest ordering the complete ZF as an artificial minigen, avoiding manual assembly of different blocks. (Optional: Clone all different ZFs into proper bacterial expression plasmids; expressed ZF must be purified and dissociation constant \(K_d\) (see Note 3) can be determined by electromobility-shift assay (EMSA), gel shift assay, enzyme-linked immunosorbent assay (ELISA) surface plasmon resonance or a BIAcore system (see Note 4)).

4. Clone the selected ZF into a mammalian plasmid vector or viral (stable) expression system (see Note 5).

Transduction of cells

1. Day -1: Seed 3.5-4 million HEK293T cells in 10 dishes along with 10 mL of medium (3 dishes per each plasmid to be transduced (in this example: 9 plates, corresponding to three constructs) plus one additional dish as control).

2. Day 0: Refresh medium between 2 and 3 hours before transfection using 5mL of HEK293T medium.

3. Day 0: Prepare the transfection mixture: 200 µL mQ water, 7.5 µg plasmid mix (2.5 µg of pMD2.G plasmid, 5.0 µg of pMDLg/pRRE plasmid); fill up to 450 µl with mQ then add 50 µl CaCl\(_2\) (2.5 M) drop wise and mix, leave at room temperature (prepare three times this mix, one per dish to be transduced).

4. Day 0: Into a tube containing 1500 µl of HBS 2x solution, add the complete (three times mixture) transfection mixture (see Note 6); incubate 20-30 min and add a final volume of 1 mL of the solution to each HEK293T cell dishes. Swirl the plates and put them at 37°C 5% CO2 incubator (see Note 7).

5. Day 1: Refresh the medium of treated HEK293T cell using 5 mL of pre-warmed medium and seed host cells (A2780) in T75 flask using 700.000 cells in 15 mL of medium.

6. Day 2: Collect HEK293T cells supernatant medium in a single tube according to the plasmid to be transduced (± 5 ml virus medium per dish, three dishes per plasmid), and add 5 ml of new medium to the each HEKEK293T cells dishes and put them back in the virus 37°C CO2 incubator.

7. Day 2: Centrifuge all collected virus medium to spin down the debris and cells (1000xg for 10 min) and filter each supernatant trough a 0.45 µm SFCA filter.

8. Day 2: Per each HEK297 cell dish used previously (5mL), and in independent tubes (1 tube per construct to be transduced) 400 µmL FCS (final conc. 10%) and 30 µl polybrene (final conc. 6 µg/ml). Transfer the filtered virus containing media to the correct amount of solution (FCS+Polybrene) and mix gently.

9. Day 2: From the appropriate A2780 cell flask, aspirate the medium and add 7 mL of the corresponding virus medium (and save the remaining virus medium at 4°C). Leave the cells at 37°C incubator for 8 hours; at the end of which, the virus medium of the A2780 cell flask must be replaced with previously saved virus medium. Finally put back the cells in the virus 37°C CO\(_2\).

10. Day 3: Repeat the procedure made on day 2 to the HEK293T cell supernatant and host A2780 cells. The virus producer cell HEK293T, can be discarded after the second virus collection.

11. Day 4: Refresh medium of the host cells.
2. Sorting of successfully transduced GFP positive cells

Day 5+: In order to select the transduced cells, take advantage of the GFP protein expressed along with the Zinc-Finger protein from the pMX-CD54-Opt31 plasmids (see Note 8).

1. Discard the medium and wash the cells 3 times with pre-warmed PBS.
2. Add 500 µL TEP, spread around the flask and incubate 37°C until cells detach (see Note 9).
3. Inactivate TEP by adding 2 mL of fresh medium and resuspend cells on it.
4. Collect the cells and centrifuge at 500xg for 5 minutes.
5. Discard supernatant and resuspend the cell pellet in pre-warmed PBS.
6. Proceed to FACS sorting (see Note 10).
7. Recover sorted cells in the proper medium and seed the cells in an appropriated multi-ple well plate according to the number of recovered cells Table 1 (see Note 11).

<table>
<thead>
<tr>
<th>Cell culture plate</th>
<th>Well diameter (mm)</th>
<th>Approx. Growth Area (cm²)</th>
<th>Average cell seeding density</th>
<th>Working volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>35.8</td>
<td>9.5</td>
<td>3.0 x10⁵</td>
<td>1.9-2.9</td>
</tr>
<tr>
<td>12 well</td>
<td>22.1</td>
<td>3.8</td>
<td>1.0x10⁵</td>
<td>0.76-1.14</td>
</tr>
<tr>
<td>24 well</td>
<td>15.6</td>
<td>1.9</td>
<td>0.5x10⁵</td>
<td>0.38-0.57</td>
</tr>
<tr>
<td>96 well</td>
<td>11</td>
<td>0.95</td>
<td>1.3x10⁴</td>
<td>0.19-0.28</td>
</tr>
</tbody>
</table>

Table 1. General cell culture plates culturing characteristics.

3. DNA extraction and bisulfite conversion

1. Culture transduced cells in 6 well plates
2. Discard the medium and wash the cells 3 times with pre-warmed PBS
3. Add 200 µL TEP, spread around the well and incubate 37°C until cells detach
4. Inactivate TEP and resuspend cells by adding 2 mL of fresh medium
5. Collect the cells and centrifuge at 500xg for 5 minutes
6. Discard supernatant and save cell pellet at -80°C or proceed directly to DNA extraction
7. Incubate cell pellet at 55°C for 5-10 min
8. Resuspend pellet in 500 mL SE 1x solution
9. Add 1 µL RNase and incubate 1 h at RT
10. Add 5 µL proteinase K and 50 µL SDS 10%, mix by inverting tube several times.
11. Incubate for at least 2 h at 55°C mixing constantly by rotating upside down (see Note 12).
12. Add 222 µL (0.4 volume) NaCl 6M, shake vigorously
13. Add 777 µL (1 volume) Chloroform, shake the tube until two layer are completely mixed
14. Rotating tubes upside down for at least 20 min up to 1 h
15. Centrifuge samples at 12000 rpm 10 min at RT (see Note 13).
16. Carefully pipet out the upper layer and save in a clean 1.5 mL tube (see Note 14).
17. Measure the collected volume and add 1 volume of ice cold Isopropanol, mix until white threads of DNA form a visible clump.
18. Centrifuge samples at >8000xg 15 min at 4°C.
19. Carefully pipette out the supernatant without disturbing the DNA pellet.
20. Add 500 µl of ice cold ethanol 70% and resuspend the pellet.
21. Centrifuge >8000xg 5 minutes at 4°C, carefully decant the ethanol and leave the pellet to air dry at room temperature.
22. After all the ethanol is evaporated, add 30 µl TE buffer or mQ .
23. Quantify the DNA (e.g. by Nanodrop)
24. Storage: at 4°C or at -20°C for longer periods.
25. Use any of the commercially available bisulfite conversion kit to ensure high conversion ratio and as much as possible converted DNA recovery (see Note 15).
26. CpG sites in the converted DNA can be evaluated by Pyrosequencing or bisulfite sequencing. We suggest pyrosequencing to quantify the methylation % of each CpG site

4. Pyrosequencing
1. Proceed to PCR amplification reaction using PyroMark™ PCR kit using protocol described by manufacturer TABLE 2 (see Note 16).
2. Run up to 5 µL of PCR product in an agarose gel 1% and identify the positive amplification (see Note 18).
3. Pyrosequencing procedure is made using PyroMark™ Q24 pyrosequencer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>Cycling protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyroMark PCR Master Mix 2x</td>
<td>1x</td>
<td>94°C 10 min</td>
</tr>
<tr>
<td>CoralLoad Concentrate 10x</td>
<td>1x</td>
<td></td>
</tr>
<tr>
<td>Q-Solution 5x</td>
<td>1x</td>
<td></td>
</tr>
<tr>
<td>Primer ICAM-1 pyro tw 20µM (Note 17)</td>
<td>0.2 µM</td>
<td>94°C 30 s 45 cycles</td>
</tr>
<tr>
<td>Primer ICAM-1 pyro rv 20µM</td>
<td>0.2 µM</td>
<td>56°C 30 s 45 cycles</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>--</td>
<td>72°C 30 s</td>
</tr>
<tr>
<td>Template DNA BS 20 ng</td>
<td>1.6ng/µL</td>
<td>72°C 10 min</td>
</tr>
<tr>
<td>Total volume (after adding template DNA)</td>
<td>25 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: PCR conditions for PyroMark™ PCR kit usage.
4. Prior to pyrosequencing procedure, use PyroMark™ Assay Design software to determine the position in the PyroMark™ Q24 plate and the amount of enzyme, substrate and each dNTP needed for the assay and save the running protocol on a USB stick.
5. For each sample to be tested, dilute the sequencing primer to 0.3 µM in 25 µL.
6. Fill all the stations of the PyroMark™ Q24 vacuum workstation, with the appropriate volumes of Ethanol 70%, Denaturation solution, wash buffer and water.
7. In 0.2 mL tubes, mix 20 µL of PCR product, with 1 µL of Streptavidin Sepharose™ High Performance beads, 40 µL of PyroMark™ Binding Buffer, and 19 µL of mQ water, shake the solution at ± 1400 rpm up to 15 min.
8. Add to the previously defined wells (step 4) of the PyroMark™ Q24 plate, 25 µL of sequencing primer and place it in the PyroMark™ Q24 vacuum orkstation.
9. Place the 0.2 mL tubes in the proper position respect to the PyroMark™ Q24 plate in the PCR tube plate of the PyroMark™ Q24 vacuum workstation
10. Using PyroMark Q24 vacuum tool, aspirate all beads, and transfer the vacuum tool throw the steps 1, 2 and 3 of the PyroMark™ Q24 vacuum workstation and release the beads into the PyroMark™ Q24 plate (see Note 19).
11. Incubate the PyroMark™ Q24 plate containing sequencing primers and streptavidin-PCR product beads at 80°C for 2 mins and let it cool down at RT
12. Place the PyroMark™ Q24 plate in the PyroMark™ Q24 pyrosequencer
13. Fill the PyroMark™ Q24 Cartridge with the proper amount of enzyme, substrate and dNTPs previously determined in step 4.
14. Place the PyroMark™ Q24 Cartridge and Pyromark Q24 plate in the PyroMark™ Q24 pyrosequencer and run the protocol saved from step 4.
15. After running, determine the percentage of methylation in each step using PyroMark Assay Design software.
1. This is the key step for zinc finger design, DNase I sensitive regions might provide good targets for ZF binding, although for some DNase I non-sensitive sites effective ZFs have been designed. We advise to design and screen a panel of ZFs for the same region, as some might work in one cell line, while others are active in other lines (see for example Huisman et al., 2013).

2. The target site is divided in N pieces of 3 bp or overlapping 4 bps segments where the last base of the 4 bp block is the first base of the next block. Modules for ZF sequence design can be found online (http://www.scripps.edu/barbas/zfdesign/zfdesignhome.php) or via other references.

3. Specificity of the designed ZF can be examined by testing mutated target sites.

4. Several systems for ZF binding properties can be used as systematic evolution of ligands by exponential enrichment (SELEX), cyclic amplification and selection of targets (CAST), DNA microarrays or even bacterial one hybrid system.

5. Zinc Finger targeting efficiency could be verified by transient reporter assay or by measuring expression levels of the target gene.

6. Add the transfection mixture drop wise while, at the same time, using a mechanical pipettor and a 1mL pipet directly at the bottom, blow small bubbles into the solution to favor the dilution. Use a mechanical pipettor attached to a plugged 1- or 2-ml pipet to bubble the HBS and add the DNA/CaCl₂ mixture.

7. Because of the infectious potential of the virions being produced, special cautions should be taken regarding laboratory space chosen for this procedure.

8. Usually low cytotoxicity is observed and 2 days after transduction, infected cells can be selected.

9. A2780 cells can take some time to detach completely; be sure to wash the cells properly and to cover the whole flask with TEP, even adding up to 1 mL TEP.

10. Be aware to resuspend completely the cells to avoid needle obstructions, use the proper needle according to the size of your cells. Use the negative control cells to calibrate the Fluorescence activated cell sorter.

11. Make passages of cells while increasing the growing surface until enough cells are available for DNA extraction and analysis (we suggest to also freeze some ampuls).

12. Overnight incubation at 55°C with constant mixing will increase protease action facilitating step procedures.

13. Two colorless layers separated by third white protein layer are observed. Avoid disturbing the layers, if it happens, re-centrifuge 20 min and continue extraction.

14. Uncomplete protease reaction will leave protein fractions in the upper layer, usually connected to the intermediate layer, which during pippeting will drag the protein layer increasing contamination of final DNA.

15. We find that EZ DNA methylation-Gold kit, using 500 ng of gDNA and the alternative 2 of incubation 98°C 10´ followed by 53°C 4 hours, exert a good conversion ratio.

16. Test different cycling protocols (focusing on annealing step) in order to improve PCR amplification. Concentration can be tested and adapted depending on the amplification results; Q solution can also be obviated.
17. One of the primers must be biotinylated in order to perform pyrosequencing, in many cases an extended (non-biotinylated primer Fw or Rv) can be used; in this case, an additional universal biotinylated reverse primer must be used. This additional primer is complementary to the extended primer. We suggest a different cycling protocol is necessary for these conditions Table 3.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
<th>Cycling protocol</th>
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<td>1x</td>
<td>50°C** 30 s</td>
</tr>
<tr>
<td>Primer pyro fw 20μM</td>
<td>0.2 μM</td>
<td>72°C 30 s</td>
</tr>
<tr>
<td>Primer pyro rv1 20μM*</td>
<td>0.04 μM</td>
<td>20 cycles</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Template DNA BS 20 ng</td>
<td>1.6ng/μL</td>
<td></td>
</tr>
<tr>
<td>Total volume (after adding template DNA)</td>
<td>25 μL</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: PCR conditions for PyroMark™ PCR kit usage using not biotinitated primers

Step 2: Immediately add the biotinylated pyro rv2 primer** to a final concentration 0.16 μM and continue running for additional 20 cycles.
*extended sequence specific primer not biotinilated
**Biotionated Rv primer targeting the extension of pyro rv1
***Melting temperature between 1st and 2nd step must be adapted.

18. PCR products for pyrosequencing can be stored up to 24 h at 4°C, although we suggest using them as soon as possible due to observed decreased pyrosequencing efficiency in longer storages PCR products.

19. Special care must be taken when aspirate the bead, to ensure complete collection from tubes and when releasing of the beads exactly on the correct well of the PyroMark™ Q24 plate. We suggest keeping suspended the vacuum tool in the exact position before turning off the vacuum source.
REFERENCES


