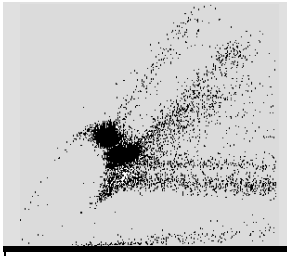

THE GOODELL LABORATORY

Author	Aysegul Verim	February 29,2012
Title	**Bisulfite Sequencing**	
Introduction	This protocol describes the use of bisulfite sequencing to identify methylated cytosines in the CpG islands of the gene of interest.	
Materials	1. PureLink Genomic DNA Mini Kit (Invitrogen) 2. Imprint DNA modification Kit (Sigma) 3. Taq Polymerase 4. Grant`s protocol buffer mix (MM2-optimized): Ammonium sulfate 16 mM, TRIS 67 mM pH 9, Mg 2 mM final, dNTP 0.125 mM final 5. Primers 100 nM final It is important to use Taq polymerase (not high fidelity proof reading enzymes). NEB Taq is recommended. PCR should give a single clean band. Use Invitrogen TOPO TA cloning kit to clone fresh PCR products in the sequencing vector. (K457540- TOPO TA Cloning Kit 40 rxn) You can save the money by halving the amount of reagents for the ligation and by splitting the competent cells in half before transformation.	
Protocol		*Notes*
1.	Sort SP^{KSL} cells into an ependorf tube	*At least 10.000* SP^{KSL} *cells.*
2.	Spin down the cells at 2000 rpm for 10 minutes at $4^{o}C$.	

3.	Remove the media and add 200 µl of PBS and follow the genomic DNA isolation kit protocol for the mammalian cells. Elute the DNA into 25-30 µl of elution buffer.	
4.	Use Imprint DNA modification kit from Sigma for bisulfite conversion. Follow the two-step modification procedure. Elute the DNA into 20 µl of elution solution.	*Use 20 µl of eluted DNA and add 4 µl of BSA (as a carrier- 0.5 mg/ml)*
5.	Use genome browser to identify the CpG island sequence. Then put this sequence into MethPrimer program in order to convert C`s into T. Design two primer sets for the nested PCR. Design the primers within the CpG island. Design on bisulfite converted DNA. Nested or semi-nested 2-step design works better. Test the primers on mouse bisulfite-treated DNA for optimal PCR conditions using gradient PCR.	*The PCR product should not be biiger than 350 bp. Primers designed by the Methprimer program could be used as an inner set of primers. Primers not on CpG.*

6.	Use 1-2 µl of bisulfite treated DNA for the nested PCR. For the first PCR reaction: By using optimised buffer: MM2/TQ2 mastermix 5X : 20% ~ 4 µl Taq polymerase 5U/ µl: 1% ~ 0.2 µl Mix at room temperature. Let the oligo bind the enzyme. dH2O: 80% ~15 µl Mix, transfer on ice and keep on ice from now on. Primer forward: 10 µM 1% ~ 0.2 µL (final 0.1 µM) Primer reverse: 10 µM: 1% ~ 0.2 µL (final 0.1 µM) Aliquot in PCR tubes or wells (16-25 µL per well). Keep on ice. Heat up the PCR block to 95C Transfer the PCR tubes or plate from ice to the hot block. Start the cycling: Initial denaturation 95C 5 min Cycle denaturation 94C 1 min Annealing/extension 60C 1 min (40 cycles) If the annealing temp. needs to be below 60C, add an extension step of 72C for 10 secs.	*Use 1-2 µl of DNA, if that does not work then increase the amount of DNA.* *TQ2: Hairpin inhibitory oligo TQ21 to prevent unspecific action of Taq polymerase at temperatures below 50°C (extension of misprimed products).The sequence is as follows:* *GCGGTCGGCTCGGGGCATTCT TAGCGTTTTGCCCCGAGCCGAC CGC* *The final concentration is 12 nM or even less if it still inhibits your PCR.*

7.	For the second PCR reaction, By using optimised buffer: MM2 mastermix 5X : 20% ~ 4 µl Taq polymerase 5U/ µl: 1% ~ 0.2 µl Mix at room temperature. Let the oligo bind the enzyme. dH2O: 80% ~15 µl Mix, transfer on ice and keep on ice from now on. Forward (nested) 10 µM : 1% ~ 0.2 µL (final 0.1 µM) Reverse (nested) 10 µM: 1% ~ 0.2 µL (final 0.1 µM) Aliquot in PCR tubes or wells (16-25 µL per well). Keep on ice. Add PCR1 as little as possible 0.2 µL Heat up the PCR block to 95C Transfer the PCR tubes or plate from ice to the hot block. Start the cycling: Initial denaturation 95C 5 min Cycle denaturation 94C 1 min Annealing/extension 60C 1 min (45 cycles to exhaust all biotinylated primer) If the annealing temp. needs to be below 60C, add an extension step of 72C for 10 secs.	
8.	Run the second PCR reaction on the 1-1.2% agarose gel with 1kb plus ladder. Purify the PCR product from the gel.	
9.	Use TOPO® TA cloning kit for ligation. Follow the protocol, mix 2µl of gel purified PCR product with 3µl of salt solution and 1 µl of vector and incubate at least for an hour at RT.	
10.	Transform 3 µl of the reaction into OneShot Top10 competent cells.	

11.	For transformation: Thaw cells on ice. Incubate DNA with competent cells for 10 minutes. Heat shock for 45 seconds at 42^{o}C. Recovery on ice for 2 minutes. Add 200 µl SOC, incubate in a 37^{o}C shaker for 45 minutes. Plate 200 µl of each transformants onto LB plates containing 100 µg/ml Spectinomycin.	
12.	From Grant`s Protocol: Pick 24 colonies from the plate and grow them in LB containing ampicillin. Transfer the bacteria in a 96-well plate with 50 µl of LB-ampicillin (100 µg/ml). Incubate the plate without shaking overnight at 37 C. Or do colony PCR in 96 well plate. For PCR use 1 µl of bacterial cultures or directly do colony PCR by using PCR4-217 F and R (60C annealing) primers. Run the PCR products on the gel to check for the correct size of inserts. (217+the size of the PCR product) Products containing single bands can be directly sequenced with M13R or M13F primers. Prefer M13R.	PCR4-217F TGTGGAATTGTGAGCGGATA PCR4-217R GTTTTCCCAGTCACGACGTT
13.	Use Sequencer program to compare the sequence with the converted sequence and see if C`s in CpG island remained as C or converted into T. If they are converted into T then they are unmethylated and if they remained as C, then they are methylated.	
14.	From Grant`s protocol: The sequences can be directly analyzed by QUMA.	

References.

1. Li LC and Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002 Nov;18(11):1427-31.