Methylation detection by PCR

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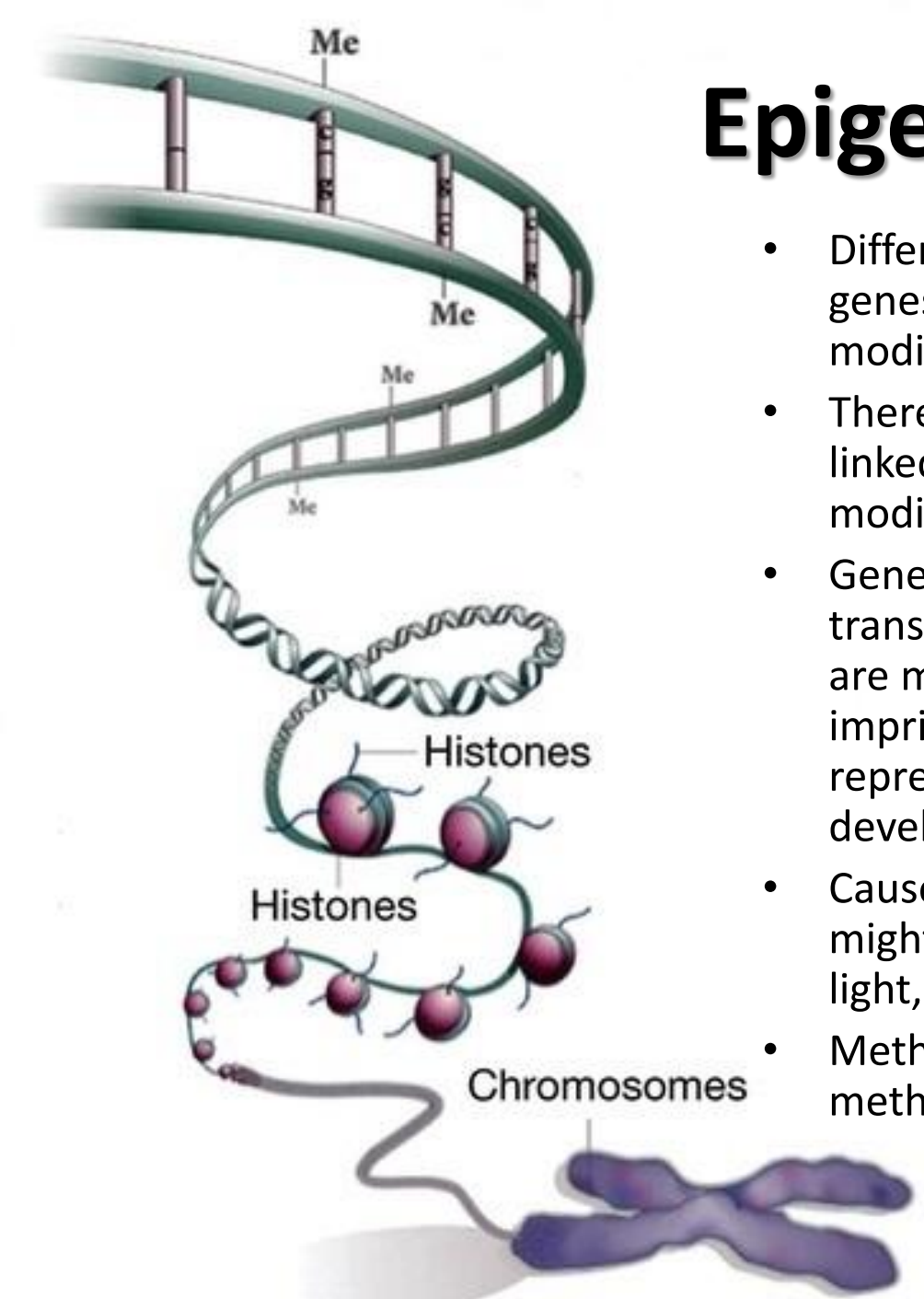
PCR training

June 13-17, 2016



http://www.nature.com/nbt/journal/v31/n10/fig_tab/nbt.2703_F4.html

Epigenetic imprinting

- Different cells have different clusters of active genes – realisated partly by epigenetic modifications;
- There are two major epigenetic modifications linked to DNA: methylation and histon-modifications;
- Gene promoter methylation entails gene transcription repression (60-90% CpG islands are methylated in mammals) – e.g. genomic imprinting, X chromosome inactivation, repression of repetitive elements, cancer developement;
- Causes of abnormal promoter methylation might be: smoking, chemicals, vitamins, UV light, different nutritients;
- Methyltransferase activity maintains correct methylation.

Methylation Specific PCR

- In general, Methylation Specific PCR (MSP) and its related protocols are considered to be the most sensitive when interrogating the methylation status at a specific locus.
- DNA denaturation and bisulfite conversion processes are consolidated into one simple step.
- Following a short on-column cleaning, converted DNA is ready for downstream PCR applications.
- Differentiation between methylated and unmethylated strands is based on methylation-specific primers.

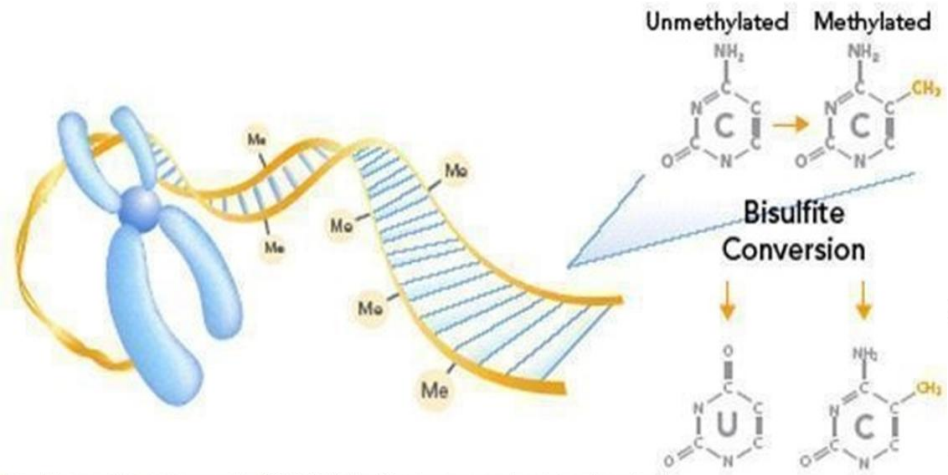
Outline of procedures

DNA denaturation and bisulphite conversion

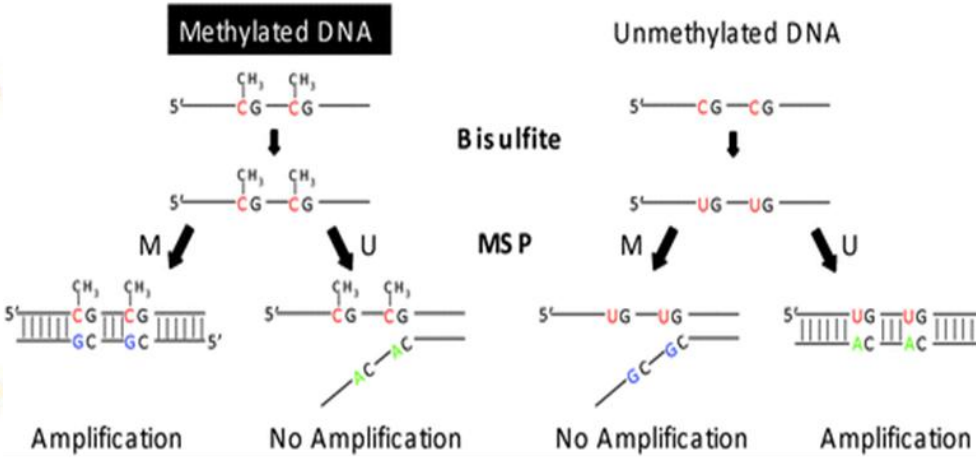
Desulphonation

MSP

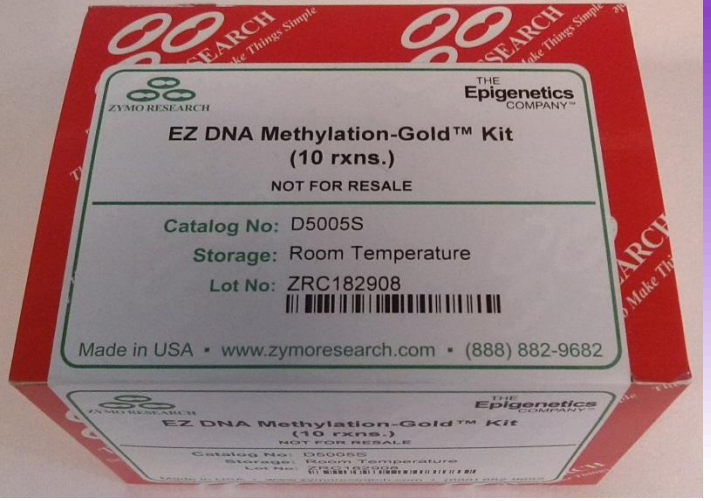
Agarose gel documentation



http://cancer-inbody.blogspot.hu/2012/11/bulsulfite-dna-sequencing-determines.html



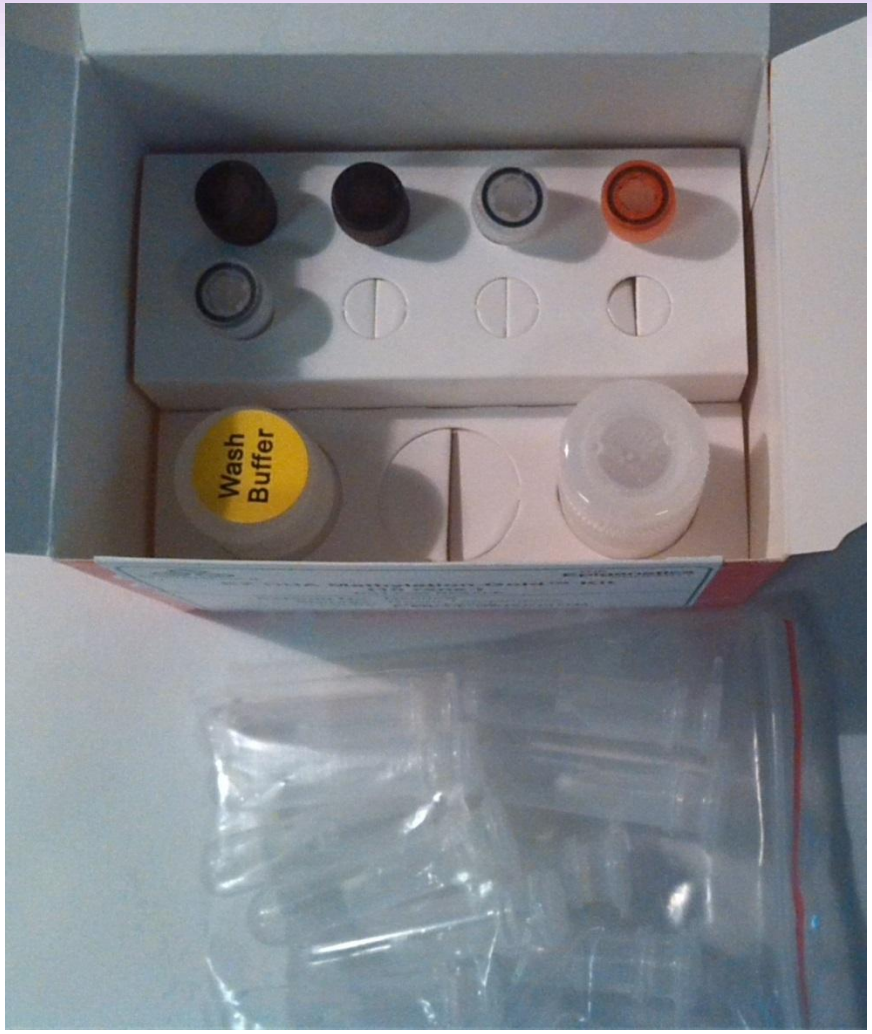
http://pubs.rsc.org/en/content/articlelanding/2009/lc/b821780g/unauth#!divAbstract



Methylation PCR protocol 1.

- **Materials required:**

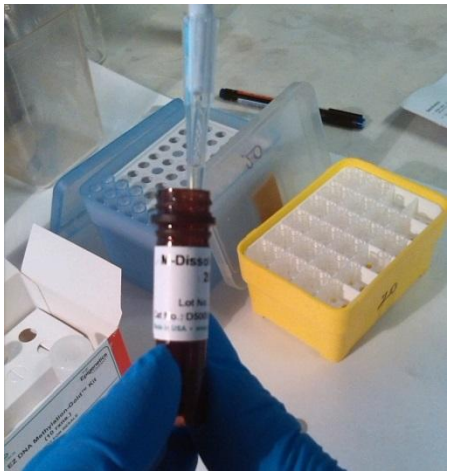
- EZ DNA Methylation-Direct Kit (ZYMO RESEARCH)
- Dynazyme polymerase and Optimized buffer for Dynazyme polymerase
- DNase free water
- automatic pipettors (100µl, 1000µl)
- 200µl PCR tubes
- PCR apparatus

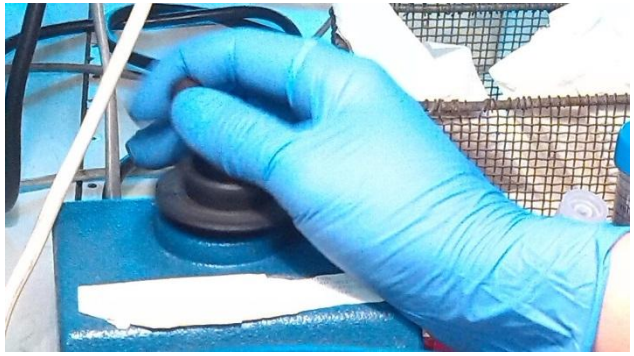


CT Conversion Reagent:

- powder reagent
- 900µl water
- 300µl M-Dilution Buffer
- 50µl M-Dissolving Buffer

- Mix thoroughly by vortex and by hand shaking for 10 minutes.
- Add 160µl M-Reaction Buffer and shake it for an other 1 minute.
- Storage: room temperature overnight/ 4°C for one week/ -20°C for a month





Methylation PCR protocol 2.

Bisulfit conversion

Conversion mix:

- 20µl DNA (50pg – 2µg, suggested amount: 1µg) RKO/SW480/A549 cell lines
- 130µl CT Conversion Reagent

Mix well, centifuge briefly

PCR conditions:

98°C 8min

53°C 3,5h

4°C 20h (max.)

Purifying converted DNA:

- add 600µl M-Binding Buffer to the filter
- add the sample and mix by inverting
- centrifuge at maximum speed for 30s
- discard the flow-through
- The converted DNA is now on the filter
- Wash with 100µl M-Wash
- Add 200µl M-Desulphonation Buffer to the column and incubate on room temperature for 15-20min
- Wash with 200µl M-Wash Buffer twice.

Elution:

- Place the filter in a 1,5ml clean tube.
- Add 10µl M-Elution Buffer directly to the matrix.
- Centrifuge on maximum spin capacity for 30s – the clean DNA is now in the eluate.

- **Store under** -20°C

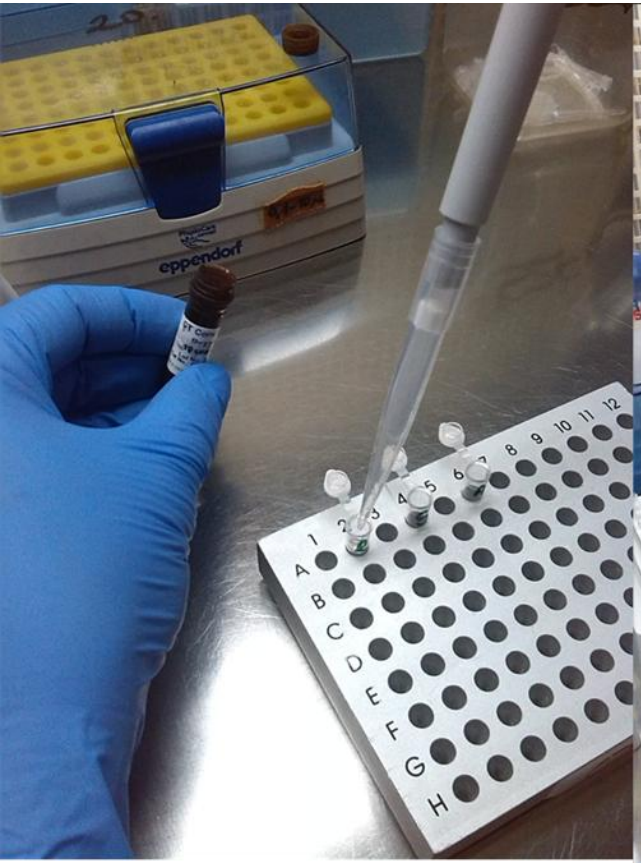
Bisulfit conversion

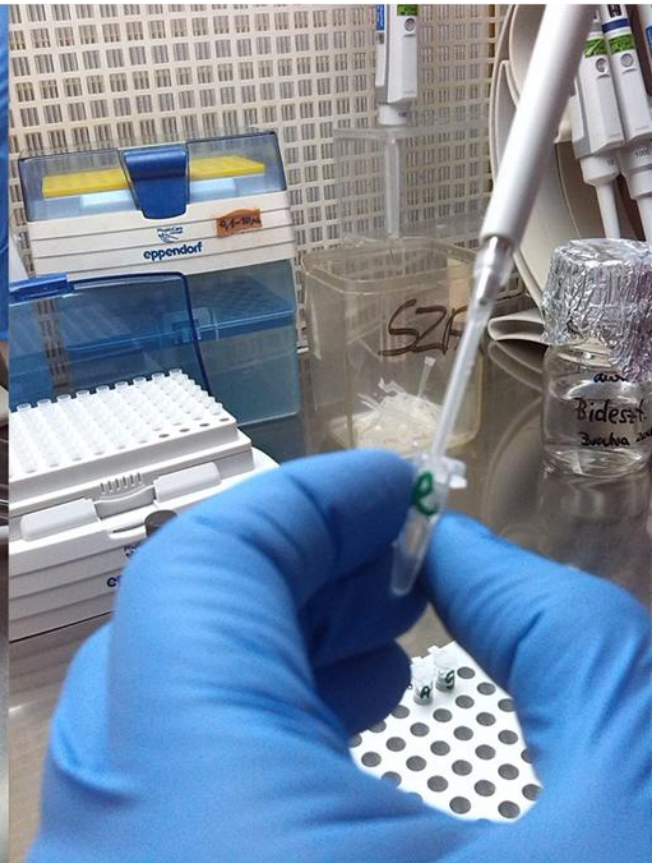
- **Conversion mix:**
  - 20µl DNA (50pg – 2µg, suggested amount: 1µg)
  - 130µl CT Conversion Reagent

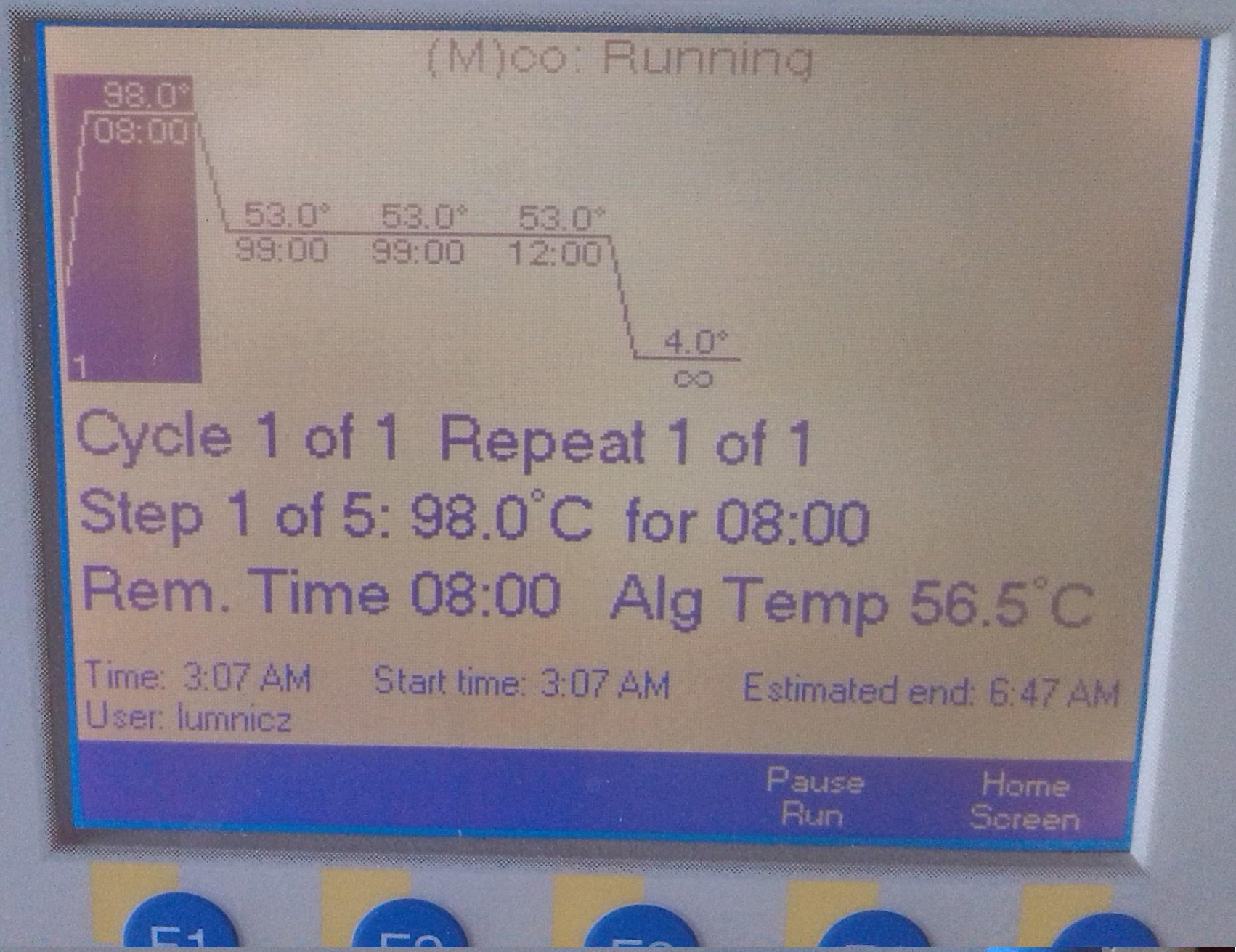
Mix well, centrifuge briefly

PCR conditions:

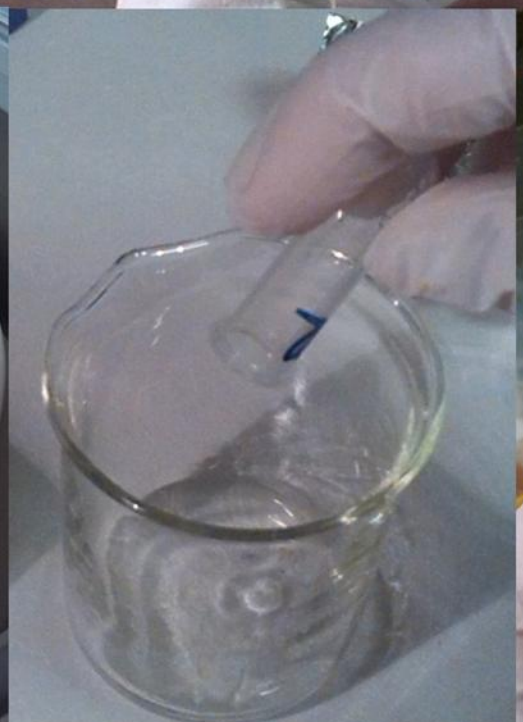
98°C 8min
53°C 3,5h
4°C 20h (max.)





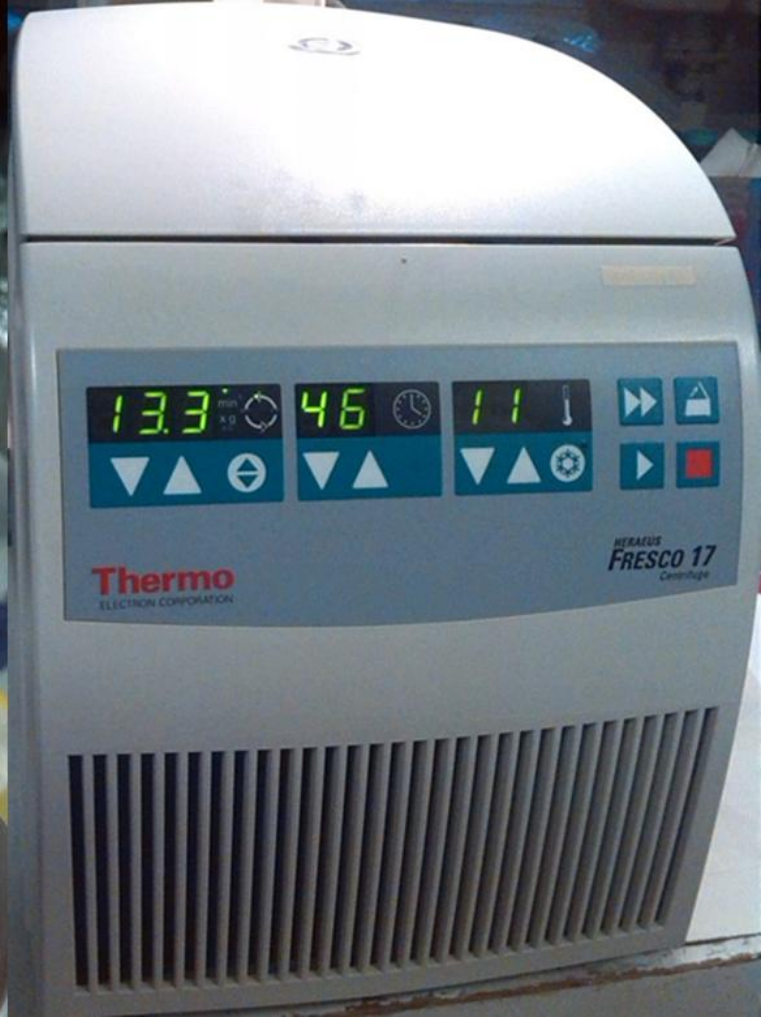

(M)co: Running
98.0°
08:00
53.0°
99:00
53.0°
99:00
53.0°
12:00
4.0°
∞
1
Cycle 1 of 1 Repeat 1 of 1
Step 1 of 5: 98.0°C for 08:00
Rem. Time 08:00 Alg Temp 56.5°C
Time: 3:07 AM
Start time: 3:07 AM
Estimated end: 6:47 AM
User: lumnicz
Pause Run
Home Screen


M-Binding Buffer
7 ml
Lot No: ZRC178312
Cat No.: D5005-3-S Storage: RT
Made in USA • www.zymoresearch.com




M-Wash Buffer
(Ethanol Added)
6 ml
Lot No: ZRC177796
Cat No.: D5001-4-S Storage:
Made in USA • www.zymoresearch.com


Desulphonating
Buffer
2 ml
Lot No: ZRC175
Cat No.: D5001-5-S St
Made in USA • www.zymo



Elution:

- Place the filter in a 1,5ml clean tube.
- Add 10µl M-Elution Buffer directly to the matrix.
- Centrifuge on maximum spin capacity for 30s – the clean DNA is now in the eluate.

Store under -20°C

Methylation PCR protocol 3.

- **Methylation PCR**

- **PCR mix:**

  2,5µl Optimalized Buffer for Dynazyme DNA polymerase

  0,5µl 10 mM dNTP mix

  1,5µl 12,5 pM MLH1 primer

  (methylated, unmethylated allel-specific primers)

  0,25µl enzim (Dynazyme)

  15,25µl DNase free water

  5µl DNA

- **Agarose Gel electrophresis**

- Assemble a 3% agarose gel (3g agarose in 100ml 1xTBE buffer) as described in “Agarose gel electrophoresis protocol”
- 1xTBE puffer
- Mix 10µl DNA sample with 2µl electrophoresis dye
- Use 6µl 100bp Promega DNA ladder in the next well of the gel
- The product is around 150 bp (154 – met, 135 - unmet).

Methylation PCR

- **PCR mix:**

2,5µl Optimized Buffer for DyNAzyme DNA polymerase

0,5µl 10mM dNTP mix

1,5µl 12,5pM MLH1 primer (methylated, unmethylated allel-specific primers)

0,25µl DyNAzyme enzime

15,25µl DNase free water

5µl converted, purified DNA

- Hot start polymerases are strongly recommended as non-specific amplification is relatively common with bisulfite-converted DNA due to it being AT-rich

Primer specifity

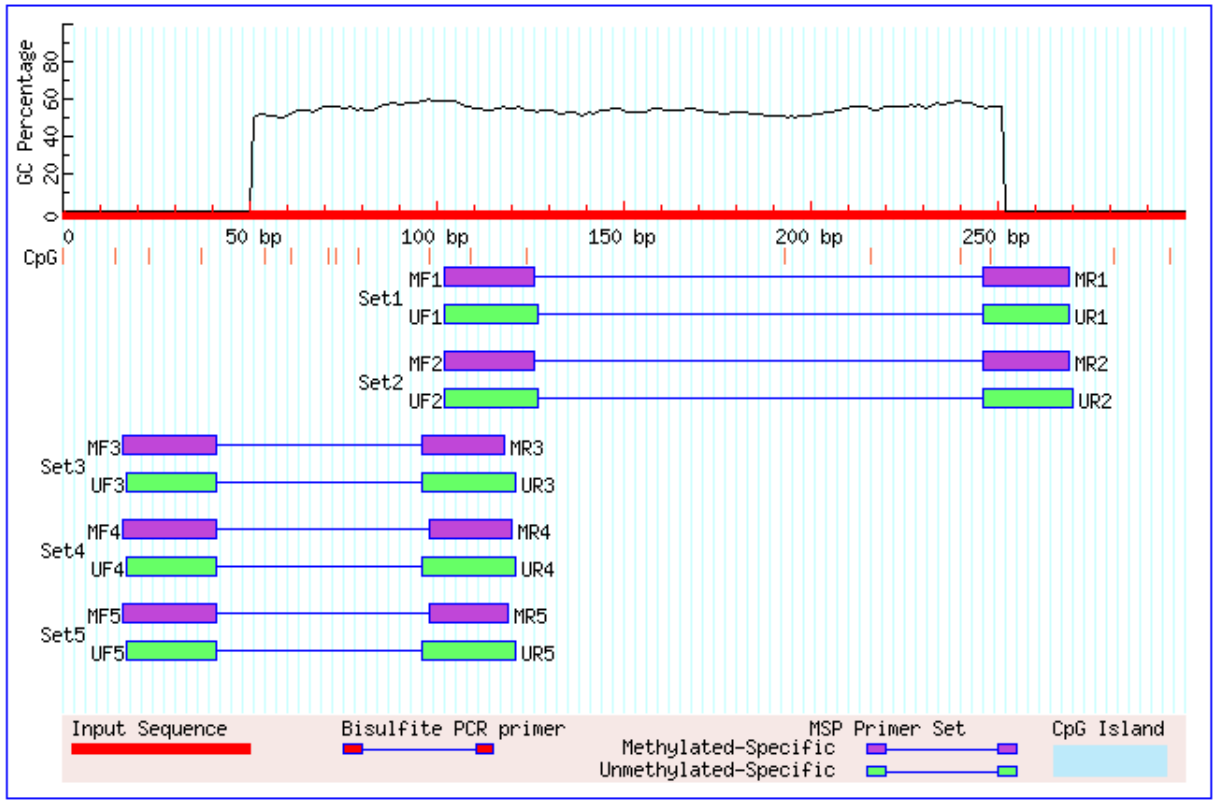
- Specific primers are neaded for methylated and unmethylated promoter PCR.
- bisulfite PCR primers need to be long (usually between 26-30 bases).
- the amplicon size should be relatively short (between 150-300 bp).
- The specificity of the assay increases with the number of CpG pairs in the primer.
- Best „fit” primers usually contain CpG at the 3'-end of the primer.
- Annealing temperatures between 55-60°C usually work well.

Designing primers with MethPrimer

- http://www.methdb.de/links.html
- http://www.urogene.org/methprimer/

MethPrimer result

Please cite MethPrimer: Li LC and Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 2002 Nov;18(11):1427-31.
PMID: 12424112



Sequence Name:
Sequence Length: 300

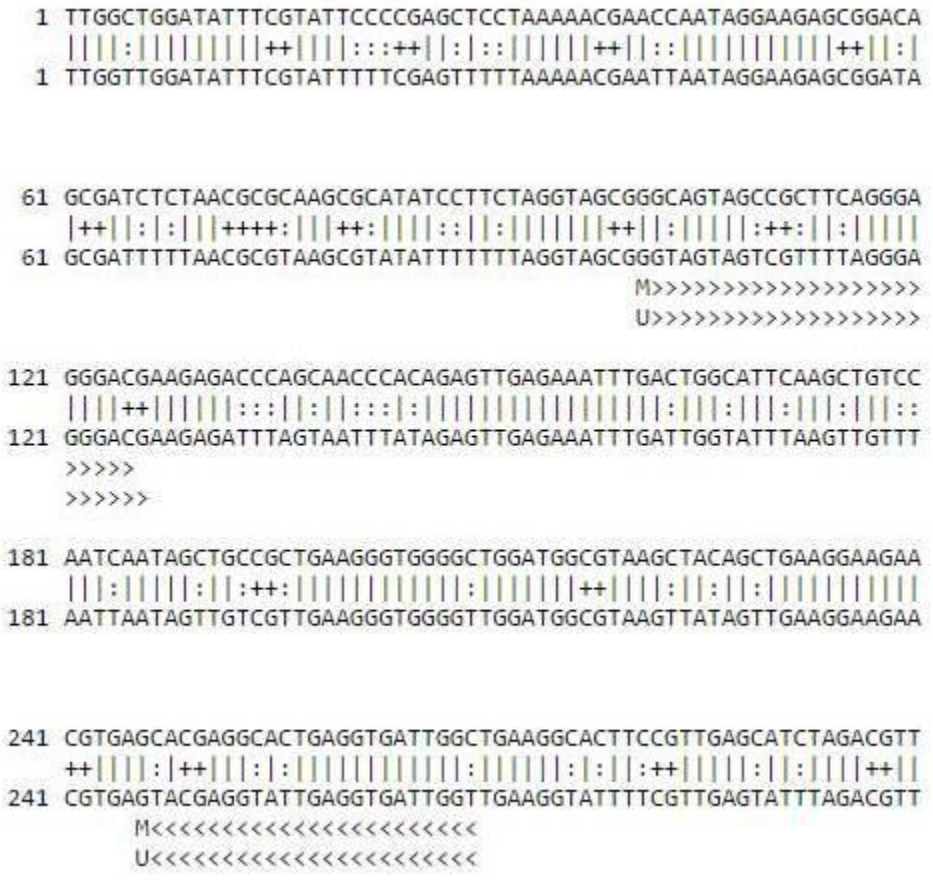
CpG island prediction results
Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.60
No CpG islands were found in your sequence
Primer picking results for methylation specific PCR (MSP)

Sequence Name: MLH1 Promoter
Sequence Length: 300

CpG island prediction results
Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.60
No CpG islands were found in your sequence
Primer picking results for methylation specific PCR (MSP)

	Primer	Start	Size	Tm	GC%	'C's	Sequence
1	Left M primer	102	24	58.71	66.67	4	GTAGTAGTCGTTTTAGGGAGGGAC
	Right M primer	269	23	59.78	60.87	4	ACCAATCACCTCAATACCTCGTA
	Product size: 168, Tm: 70.2						
	Left U primer	102	25	58.64	68.00	4	GTAGTAGTTGTTTTAGGGAGGGATG
	Right U primer	269	23	57.51	60.87	4	ACCAATCACCTCAATACCTCATA
	Product size: 168, Tm: 67.6						
2	Left M primer	102	24	58.71	66.67	4	GTAGTAGTCGTTTTAGGGAGGGAC
	Right M primer	269	23	59.78	60.87	4	ACCAATCACCTCAATACCTCGTA
	Product size: 168, Tm: 70.2						
	Left U primer	102	25	58.64	68.00	4	GTAGTAGTTGTTTTAGGGAGGGATG
	Right U primer	270	24	58.82	58.33	4	AACCAATCACCTCAATACCTCATA
	Product size: 169, Tm: 67.5						
3	Left M primer	16	25	57.37	48.00	6	GTATTTTTCGAGTTTTTAAAAACGA
	Right M primer	118	22	58.91	68.18	4	CCTAAAACGACTACTACCCGCT
	Product size: 103, Tm: 68.0						
	Left U primer	17	24	52.60	45.83	6	TATTTTTTGAGTTTTTAAAAATGA
	Right U primer	121	25	58.37	68.00	4	CTCCCTAAAACAACTACTACCCACT
	Product size: 105, Tm: 64.7						
4	Left M primer	16	25	57.37	48.00	6	GTATTTTTCGAGTTTTTAAAAACGA
	Right M primer	120	22	59.56	68.18	4	TCCCTAAAACGACTACTACCCG
	Product size: 105, Tm: 68.2						
	Left U primer	17	24	52.60	45.83	6	TATTTTTTGAGTTTTTAAAAATGA
	Right U primer	121	25	58.37	68.00	4	CTCCCTAAAACAACTACTACCCACT
	Product size: 105, Tm: 64.7						
5	Left M primer	16	25	57.37	48.00	6	GTATTTTTCGAGTTTTTAAAAACGA
	Right M primer	119	21	57.84	71.43	4	CCCTAAAACGACTACTACCCG
	Product size: 104, Tm: 67.9						
	Left U primer	17	24	52.60	45.83	6	TATTTTTTGAGTTTTTAAAAATGA
	Right U primer	121	25	58.37	68.00	4	CTCCCTAAAACAACTACTACCCACT
	Product size: 105, Tm: 64.7						

https://eu.idtdna.com/site



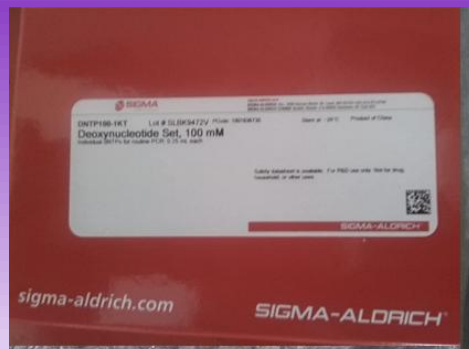
Primers used:

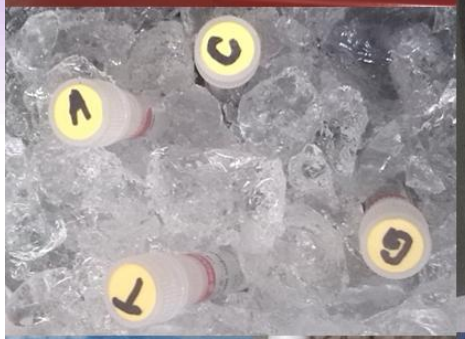
MLH1 gene promoter

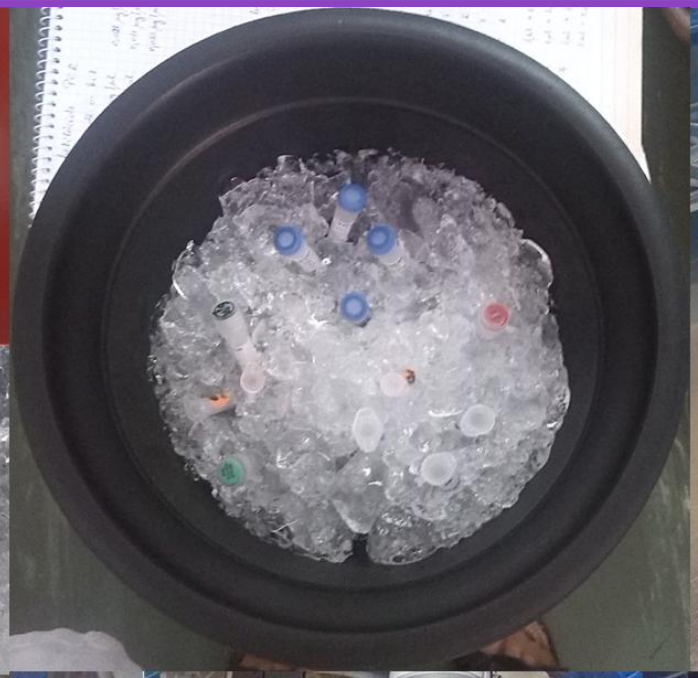
TTGGCTGGATATTTCGTATTCCCCGAGCTCCTAAAAACGAACCAATAGGA -251
AGAGCGGACAGCGATCTCTAACGCGCAAGCGCATATCCTTCTAGGTAGCG -201
GGCAGTAGCCGCTTCAGGGAGGGACGAAGAGACCCAGCAACCCACAGAGT -151
TGAGAAATTTGACTGGCATTCAAGCTGTCCAATCAATAGCTGCCGCTGAA -101
GGGTGGGGCTGGATGGCGTAAGCTACAGCTGAAGGAAGAACGTGAGCACG -51
AGGCACTGAGGTGATTGGCTGAAGGCACTTCCGTTGAGCATCTAGACGTT -1

- F M primer: 5' – GAGCGGATAGCGATTTTTAAC – 3'
- R M primer: 5' – CAACCCCACCCTTCAACG – 3'
- F U primer: 5' – AGGAAGAGTGGATAGTGATTTTTAAT – 3'
- R U primer: 5' - CAACCCCACCCTTCAACA – 3'

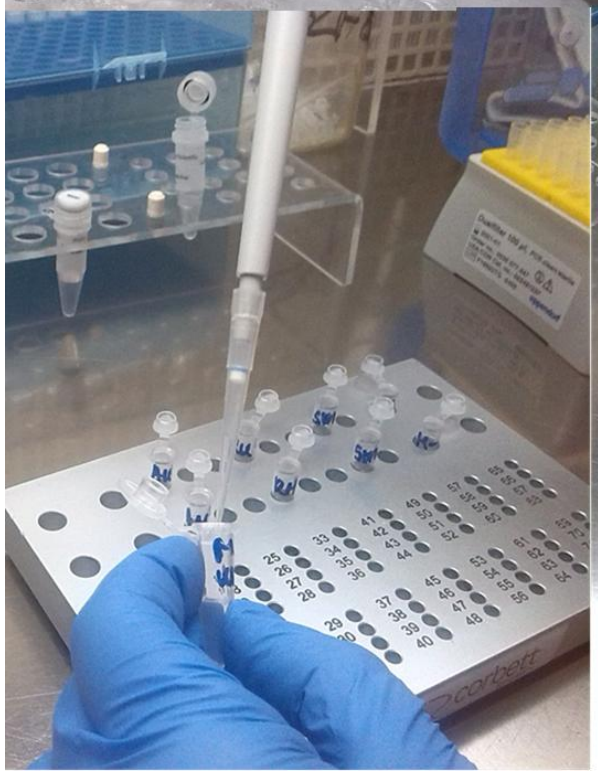
- **MJ Baek, H Kang, SE Kim, JH Park, JS Lee, Y-K Paik and H Kim:** Expression of hMLH1 is inactivated in the gastric adenomas with enhanced microsatellite instability. In. *British Journal of Cancer* (2001) 85(8), 1147–1152


Deoxynucleotide Set, 100 mM
sigma-aldrich.com
SIGMA-ALDRICH

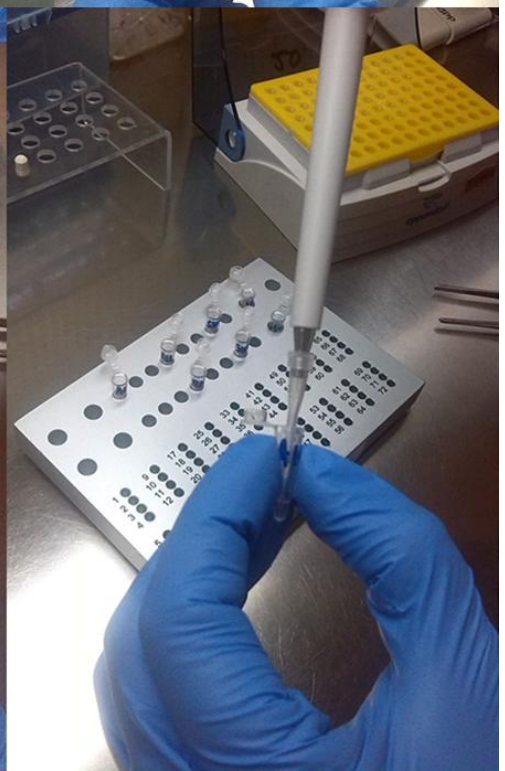

A
C
G
T

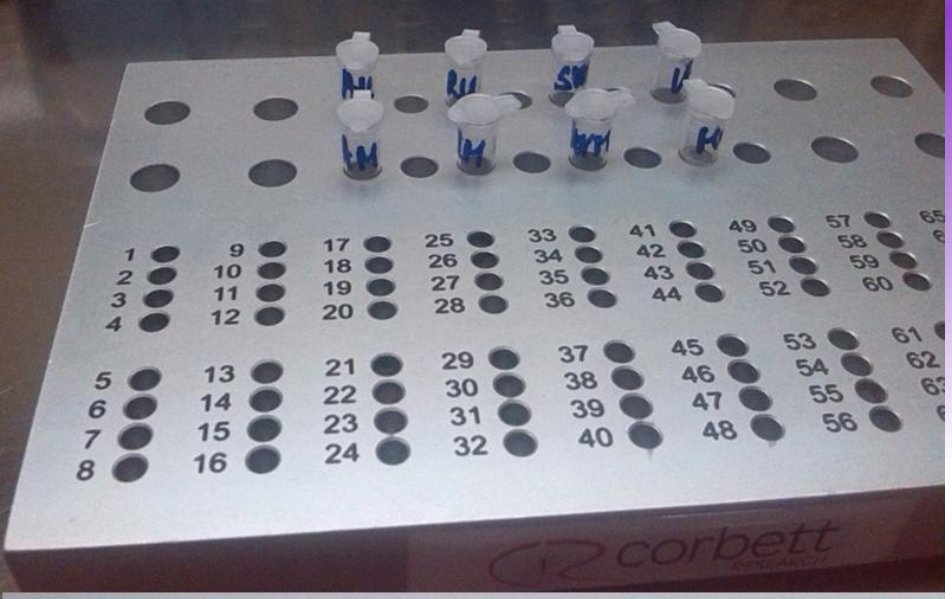



F-501L
DyNAzyme
DNA Polymerase


corbett


10





PCR settings

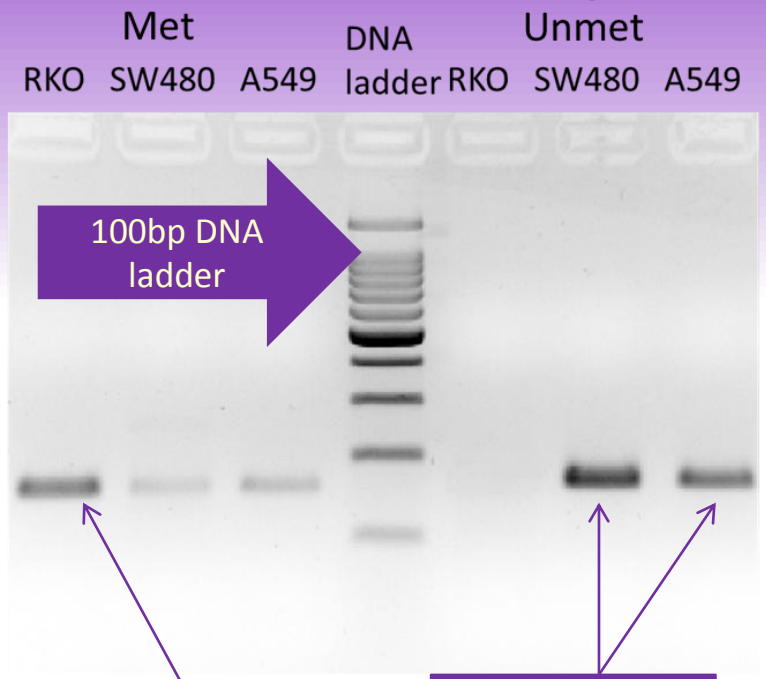
94°C 2min
94°C 30sec
60°C 30sec
72°C 30sec
} 45x
72°C 10min

Hold temperature at 4°C

Agarose Gel electrophresis

- Assemble a 3% agarose gel
  - (3g agarose in 100ml 1xTBE buffer) as described in “Agarose gel electrophoresis protocol for DNA”
- 1xTBE puffer
- Mix 10µl DNA sample with 2µl electrophoresis dye.
- Use 6µl 100bp Promega DNA ladder in the next well of the gel.
- The product is around 160bp (160bp – methylated, 165bp – unmethylated).

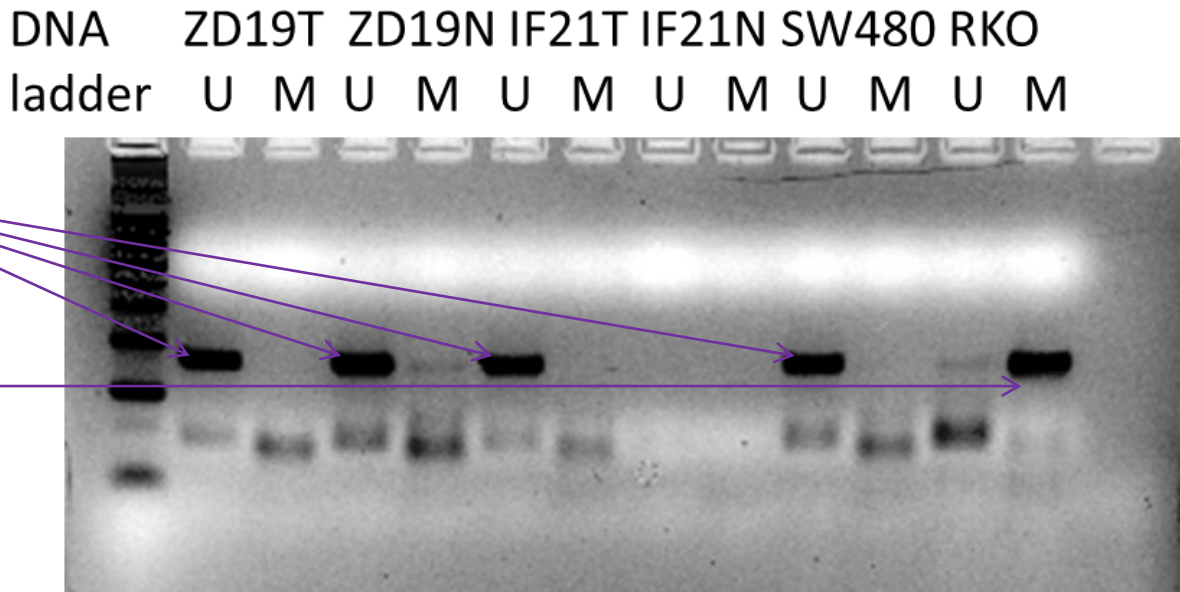
Agarose gel electroforesis



Methylation positive 160bp

Methylation negative 165bp

HNSCC patients' normal and tumor tissue samples



Evaluation of the results

- RKO cell line is a methylation positive control
- SW480 is a methylation negative control
- The -229 CpG of the MLH1 gene promoter is not methylated in A549 cell line.
- Promoter of MLH1 is not methylated at this point in tumor samples of the HNSCC patients.

Limitations of the method

- 5-Hydroxymethylcytosine does not convert by bisulfite sequencing – false positive results
- Incomplete conversion brings bias to the results – DNA denaturation is critical
- DNA degradation during conversion via depurination and random strand breaks – unspecific stripes on gel or absence of product
- Incomplete desulphonation of pyrimidine residues might compromise polymerase enzimes

Other methods for methylation research

- Direct sequencing
- Pyrosequencing
- Methylation-sensitive single-strand conformation analysis
- High resolution melting analysis (HRM)
- Methylation-sensitive single-nucleotide primer extension
- Base-specific cleavage/MALDI-TOF
- Microarray-based methods

Literature

- Bisulfite DNA Sequencing Determines Base Methylation in Epigenetics Research http://cancer-inbody.blogspot.hu/2012/11/bulsulfite-dna-sequencing-determines.html (2012.11.22 - downloaded: 2016.05.25)

- JAMES G. HERMAN*t, JEREMY R. GRAFF*, SANNA MYOHANEN*, BARRY D. NELKIN*, AND STEPHEN B. BAYLIN*t: Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA Vol. 93, pp. 9821-9826, September 1996 Medical Sciences

- http://link.springer.com/protocol/10.1007%2F978-1-61779-316-5_3#page-1

Thank You for Your Attention!