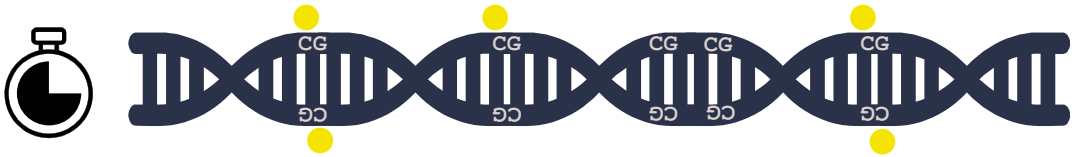
Bisulfite-converted DNA Quantity Evaluation

A multiplex quantitative real-time PCR system for the evaluation of bisulfite conversion

Sae Rom Hong
Department of Forensic Medicine
BK21 Project for Medical Science
YONSEI UNIVERSITY COLLEGE OF MEDICINE

BisQuE

01 Background: DNA methylation (DNAm)

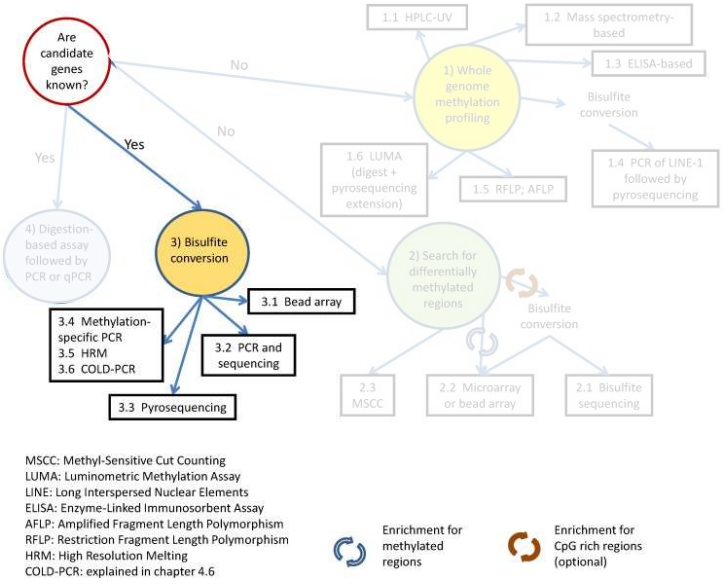


"Aging" → **Age Prediction**

"Cell differentiation" → **Bodyfluid ID**
Body tissue ID

"Environment" → **Diseases**
Habits
Lifestyle

01 Background: DNAm Analysis Methods

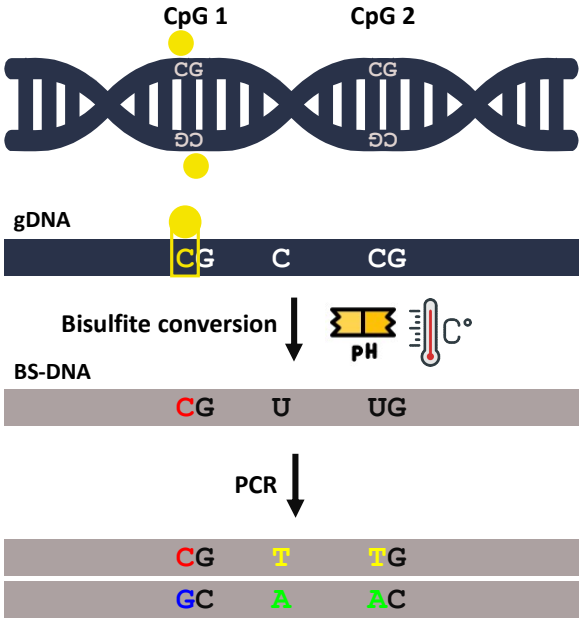


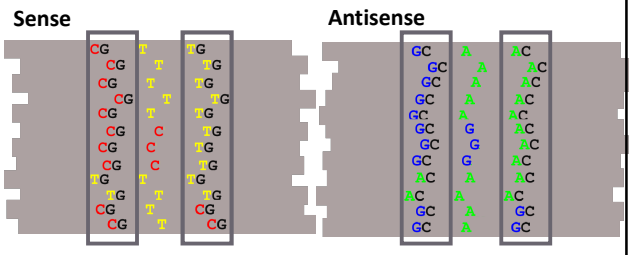
Kurdyukov and Bullock (2016) *Biology (Basel)*

- Bead array (Whole EpiGenome)
  - ✓ 27K/450K/EPIC BeadChip array
- Pyrosequencing
- Methylation-specific PCR
- High resolution melting
- Single-base extension
  - ✓ Methylation SNaPshot
- Massively parallel sequencing

Bisulfite Conversion

01 Background: Bisulfite Conversion



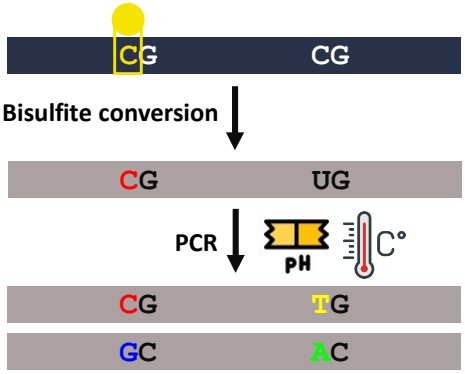


$$\frac{C}{C+T} \times 100\ (\%)$$

$$\frac{G}{G+A} \times 100\ (\%)$$

	CpG 1	CpG 2
Methyl (%)	85	17

01 Background: Bisulfite Conversion



- Harsh condition
- Degradation
- Quantity

Previous studies

- Real-time PCR
- Pyrosequencing
- Sanger sequencing
- UV spectrometry
- Gel electrophoresis
- Fluorometer
- Digital PCR
- MPS

✓ Recovery
✓ Efficiency
✓ Fragmentation (Degradation)

02 Objectives

What we want for the single assay to assess BS-DNA

1. Sensitivity and reliability
2. **Degradation level**
3. **Conversion efficiency**
4. **Recovery**
5. Presence of inhibitor

02 Objectives: Idea from quantifiler TRIO kit

User Guide: Quantifiler HP and Trio DNA Quantification Kits *Thermo Fisher Scientific*

Target	Amplicon length	Ploidy	Copy Number	Dye/Quencher
Human Target, small autosomal	80 bases	Diploid	multicopy	VIC™ dye with MGB quencher
Human Target, large autosomal	214 bases	Diploid	multicopy	ABY™ dye with QSY™ quencher
Human Male Target†	75 bases	Haploid	multicopy	FAM™ dye with MGB quencher
Internal PCR Control	130 bases	NA	Synthetic IPC template is included in the primer mix	JUN™ dye with QSY™ quencher

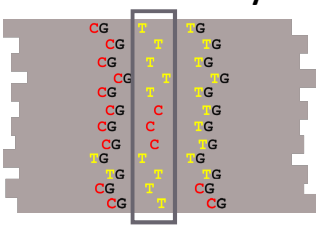
The Degradation Index is automatically calculated by the HID Real-Time PCR Analysis Software using the following formula:

$$\frac{\text{Small autosomal target DNA conc. (ng/µL)}}{\text{Large autosomal target DNA conc. (ng/µL)}}$$

1. Sensitivity and reliability
   - ✓ Multicopy (Sudmant et al. (2010) *Science*)
2. Degradation level
   - ✓ Ratio between the short- and long-sized amplicon

02 Objectives

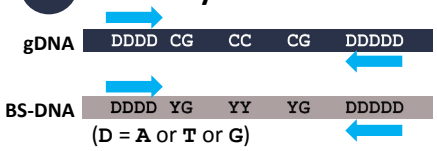
3 Conversion efficiency



- ✓ Cytosine in Non-CpG context
- ✓ Complete BS conversion: only T (U)

  Incomplete BS conversion: T + C (minor component)
- ✓ Detecting C and T

4 Recovery



- ✓ Ratio between amount of input DNA (genomic DNA) and BS-DNA
- ✓ Cytosine free primer: both gDNA and BS-DNA

5 Presence of inhibitor

- ✓ Internal positive control (IPC); artificial sequence template

02 Objectives

What we want

1. Sensitivity and reliability
2. **Degradation level**
3. **Conversion efficiency**
4. **Recovery**
5. Presence of inhibitor

What we need

1. Multicopy target
2. Short- and long-sized amplicon
3. Detecting C and T
4. Cytosine free primer
5. Internal Positive Control

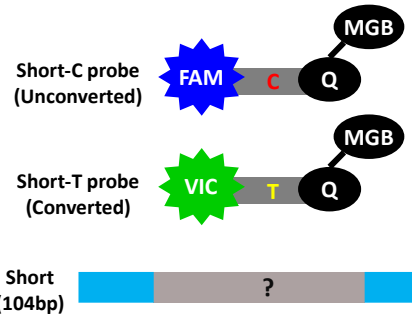
03 Real-Time PCR: Target

1 Multicopy target + 4 Cytosine free primer

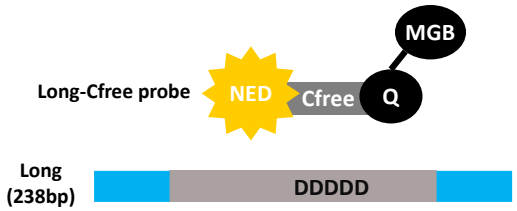
2 Short- and long-sized amplicon

✓ Short-sized (104bp): *CCDC29*

3 **Probe**: Detecting C and T



✓ Long-sized (238bp): *FLJ39739*



03 Real-Time PCR: IPC

5 IPC

- ✓ Random DNA Sequence Generator
- ✓ Non-human
- ✓ Synthetic template
- ✓ 146bp
- ✓ 500 copies

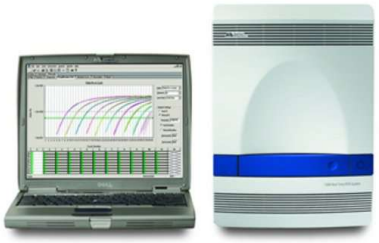
03 Real-Time PCR: Multiplex

Short size amplicon
- Multicopy + C free primer
- 104bp sized
- Detecting C **(FAM)** and T **(VIC)**

Long size amplicon
- Multicopy + C free primer
- 238bp sized
- C free probe **(NED)**

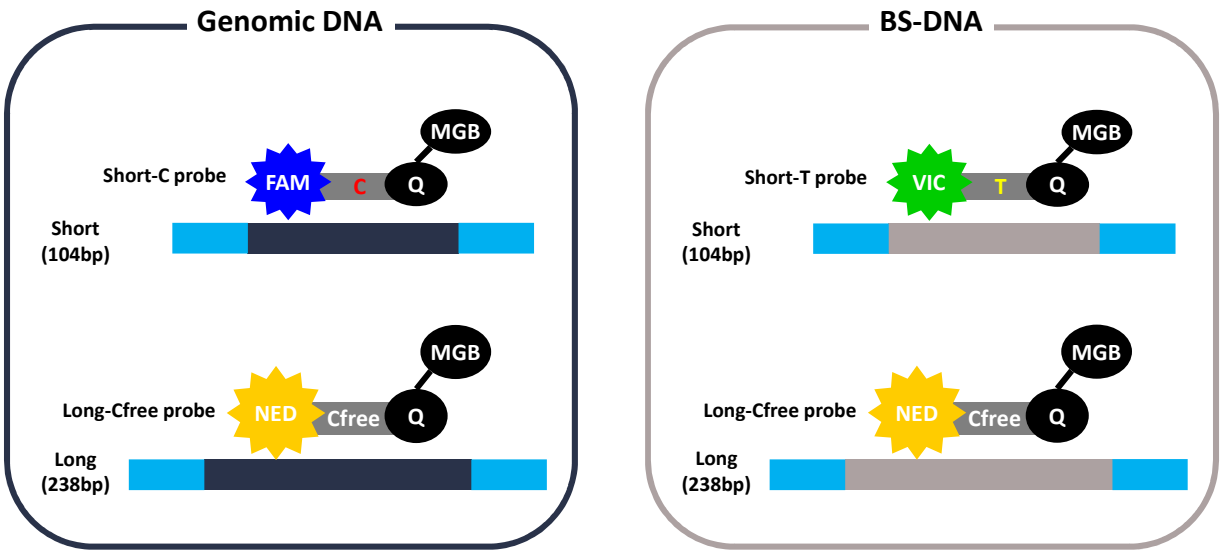
IPC
- Non-human & synthetic DNA
- Arbitrary sequence **(Cy5)**

Passive reference
- **ROX**

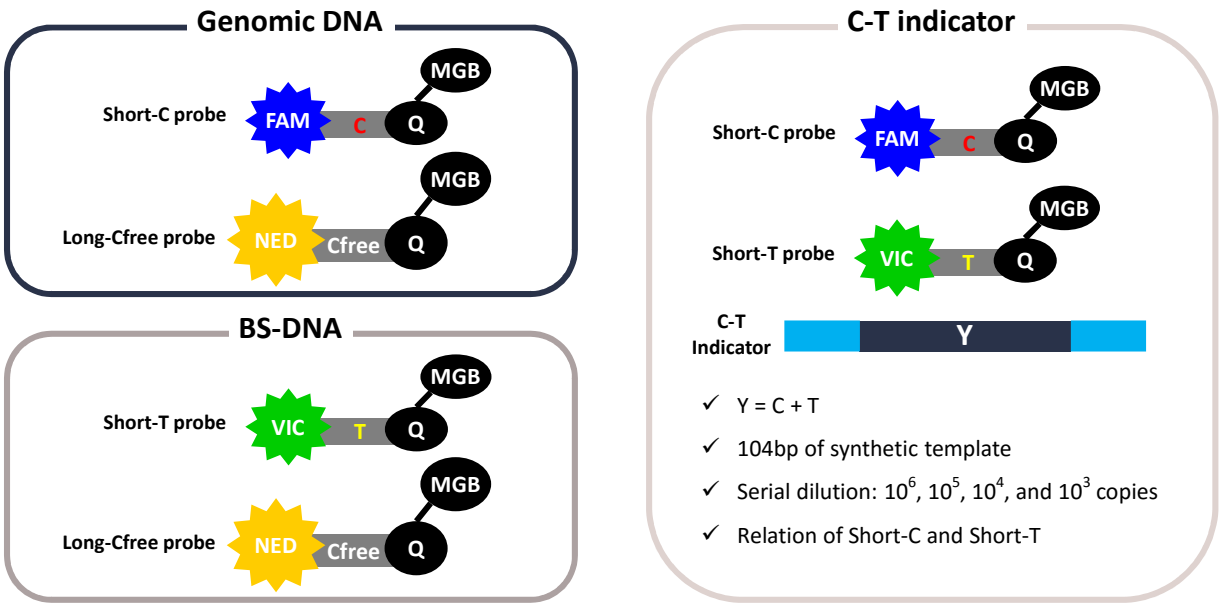


AB 7500

03 Real-Time PCR: C-T indicator



03 Real-Time PCR: C-T indicator



- ✓ Y = C + T
- ✓ 104bp of synthetic template
- ✓ Serial dilution: 10^6, 10^5, 10^4, and 10^3 copies
- ✓ Relation of Short-C and Short-T

04 Samples: gDNA and BS-DNA

gDNA

- **20 of gDNA samples** from blood
- Asian Sample Network of SNU
- IRB approval (4-2019-0707, Severance Hospital, Yonsei University)

BS-DNA

- Input: **50 ng of gDNA**
- Elution: **10 ul** of TE buffer (NEB: 20 ul following manufacturers' guide)
- **6 conversion kits**
  - ✓ EZ DNA Methlyation-Lightning kit (Zymo Research): Z-EZ
  - ✓ Premium Bisulfite kit (Diagenode): D-PB
  - ✓ MethylEdge Bisulfite Conversion System (Promega): P-ME
  - ✓ EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific): T-EJ
  - ✓ EpiTect Fast Bisulfite kit (Qiagen): Q-EF
  - ✓ NEBNext® Enzymatic Methyl-seq Conversion Module (NEB): N-NE

04 Samples

Standard

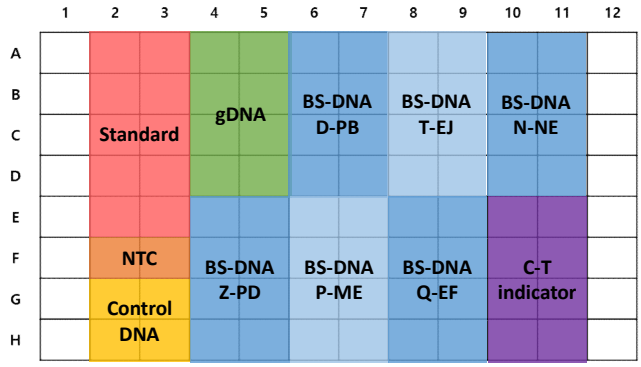
- **Standard DNA (human gDNA)** from quantifiler Duo kit (Thermo Fisher Scientific)
- 5X serial dilution: 10 ng, 2 ng, 400 pg, 80 pg, and 16 pg

C-T indicator

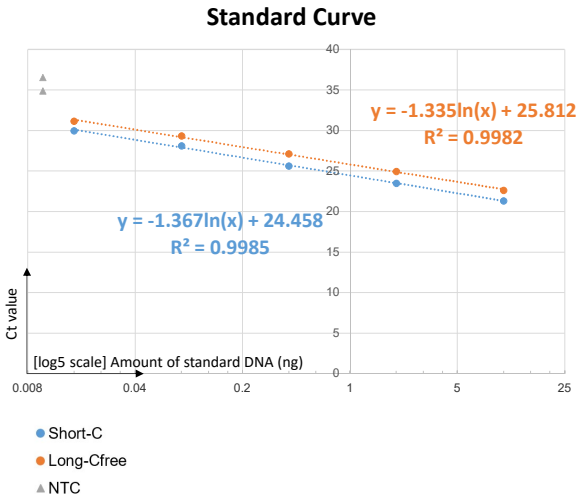
- Serial diluted: 10^6, 10^5, 10^4, and 10^3 copies

Control DNA

- EpiTect Control DNA (Qiagen)
- 1 ng



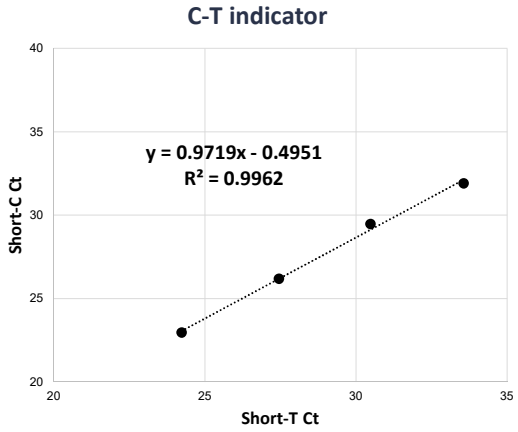
05 Result: BisQuE



Sample	Amount (ng)			Ct
	Short-C	Short-T	Long-Cfree	IPC
gDNA	4.421		4.731	27.054
BS-DNA	0.044	?	0.762	27.242

Sample	Ct value			
	Short-C	Short-T	Long-Cfree	IPC
gDNA	22.426		23.738	27.054
BS-DNA	28.784	25.537	26.175	27.242

05 Result: BisQuE



Sample	Ct value		
	Short-C	Short-T (T -> C)	Long-Cfree
gDNA	22.426		23.738
BS-DNA	28.784	25.537 -> **24.324**	26.175

Sample	Amount (ng)		
	Short-C	Short-T **(T -> C)**	Long-Cfree
gDNA	4.421		4.731
BS-DNA	0.044	**1.103**	0.762

05 Result: BisQuE

Features

3 **Conversion efficiency**

$$\frac{short - T}{short - C + short - T} \times 100\ (\%)$$

Sample	Amount (ng)		
	Short-C	Short-T **(T -> C)**	Long-Cfree
gDNA	4.421		4.731
BS-DNA	0.044	**1.103**	0.762

$$\frac{1.103}{0.044 + 1.103} \times 100\ (\%) = 96.18\ (\%)$$

05 Result: BisQuE

Features

2 Degradation level

$$\frac{BS-DNA(short/long)}{gDNA(short/long)}$$

- ✓ Multicopy
- ✓ Compensating variance

Sample	Amount (ng)		
	Short-C	Short-T **(T -> C)**	Long-Cfree
gDNA	4.421		4.731
BS-DNA	0.044	**1.103**	0.762

$$\frac{(0.044+1.103)/0.762}{4.421/4.731}=1.610$$

05 Result: BisQuE

Features

4 Recovery

$$\frac{2\times BS-DNA}{gDNA}\times 100\ (\%)$$

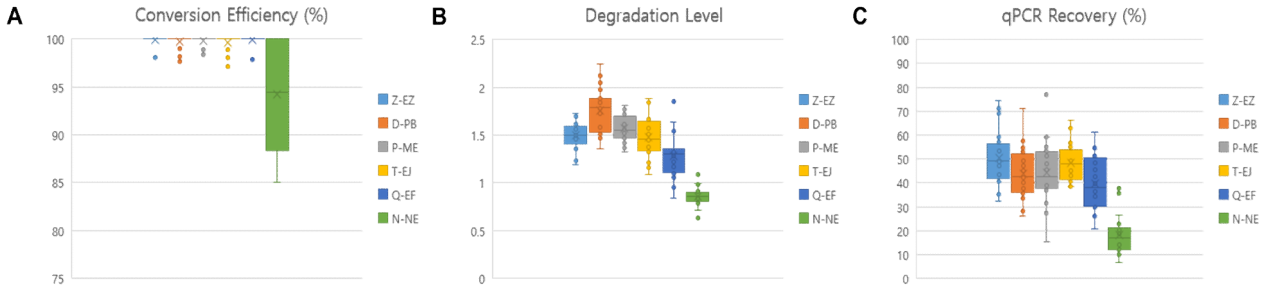
- ✓ gDNA: sense and antisense strand
- ✓ BS-DNA: sense strand
- ✓ Short amplicon
- ✓ ×2 for N-NE (20ul elution)

Sample	Amount (ng)		
	Short-C	Short-T **(T -> C)**	Long-Cfree
gDNA	4.421		4.731
BS-DNA	0.044	**1.103**	0.762

$$\frac{2\times(0.044+1.103)}{4.421}\times 100\ (\%)=51.86\ (\%)$$

05 Result: Kit performance

Kit	Z-EZ	D-PB	P-ME	T-EJ	Q-EF	N-NE
Conversion Efficiency (%)	99.90	99.74	99.78	99.61	99.89	94.24
Degradation Level	1.495	1.762	1.577	1.479	1.279	0.857
Recovery (%)	50.58	43.79	44.42	48.39	39.65	18.24



05 Result: IPC

Kit	Z-EZ	D-PB	P-ME	T-EJ	Q-EF	N-NE
Conversion Efficiency (%)	99.90	99.74	99.78	99.61	99.89	**94.24**
Degradation Level	1.495	**1.762**	1.577	1.479	1.279	**0.857**
Recovery (%)	**50.58**	43.79	44.42	48.39	39.65	**18.24**
Average IPC Ct	27.420	27.513	27.445	27.382	27.514	27.676

**gDNA Average IPC Ct: 27.458*

06 Conclusion

- Development of BisQuE system for both gDNA and BS-converted DNA
  - ✓ Short-C, Short-T, and Long-Cfree
  - ✓ Conversion efficiency + Degradation level + Recovery

- Most kits showed more than 99% of conversion efficiency, except N-NE.
- Recovery rates of kits were similar, Z-EZ showed the highest, except N-NE.
- N-NE showed the lowest degradation level due to its' chemistry. It is recommended when samples are severely degraded.
- There is no significant inhibition in BS-DNA.

- **BS-DNA input should be considered before the DNAm analysis to guarantee the accuracy of downstream analysis.**

07 Acknowledgement

- This research was supported by the National Research Foundation of Korea (NRF) funded by the Korean government (NRF- 2019R1F1A106382712).
- Brain Korea 21 Project for Medical Science
- Asian Sample Network