Translating Nanotechnology and Microfluidics for Analysis of DNA Methylation

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Methylation As a Cancer Biomarker

DNA Methylation contributes to the progression of carcinogenesis by silencing of tumor suppressor genes


Abnormal epigenetic changes appear to be an early event before detection of genetic mutations. Thus, detection of promoter methylation is a promising approach for early diagnosis of cancer.
Current Method for Promoter Methylation Detection

**Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands**

(DNA methylation/tumor suppressor genes/p16/p15)

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Advantages: High sensitivity and specificity

Limitations: • Still not sensitive enough to reliably detect methylated DNA in body fluids such as serum, sputum, stool and urine (requiring nested PCR, digital PCR).
• Tedious and labor-intensive process which is not amenable for routine clinical utilizations.
• Sub-optimal efficiency in recovery of circulating DNA
MS-qFRET: Methylation-Specific QD-FRET for DNA Methylation Detection

Methylated DNA

5'—C—CG—CG—
—G—GC—GC—5'

Unmethylated DNA

5'—C—CG—CG—
—G—GC—GC—5'

Bisulfite Conversion

5'—U—CG—CG—
+—G—GC—GC—5'

5'—U—UG—UG—
+—G—GU—GU—5'

PCR with labeled methylation specific primers

biotin
fluorophore

No Product

Add quantum dots

QD

FRET

hv1
QD Excitation

hv2
Fluorophore Excitation)

No QD-FRET

(hv1, hv2

( Bailey et al., Genome Research, 2009; Bailey, Methods, 2010; Zhang, Theranostics, 2012)
Quantum Dot-Fluorescence Resonance Energy Transfer (QD-FRET) DNA Nanosensor

1. Reporter probe
   - Cy5

2. Capture probe
   - Biotin

3. Streptavidin-conjugated QD

Excitation
- (488 nm)

Emission
- (QD) (605 nm)
- (Cy5) (670 nm)

FRET

(Zhang et al., Nat Mater, 2005)
Single-molecule/nanoassembly Detection

1. Reporter probe
2. Capture probe
3. Streptavidin-conjugated QD

Target DNA
Sandwiched hybrid
Nanosensor assembly

In the presence of targets
In the absence of targets

1. Cy5
2. biotin
3. QD

FRET
No FRET

Photon counts / ms

Time (s)

605QD

Cy5

No FRET
Cy5

605QD
Quantitative Analysis of DNA Methylation Detection with QD-FRET Nanosensor

![Graph showing fluorescence intensity vs. wavelength and q-score vs. methylated DNA percentage.](image)
DNA Methylation Detection with QD-FRET Nanosensor

Detection limit:
- Detect methylated DNA in the presence of 10,000 excess of unmethylated alleles
- 15 pg DNA (5 genomic equivalents)
Cancers comprise heterogeneous populations of cells at primary and metastatic sites.

DREAMing uses quasi-digital detection and precise melt curve analysis to distinguish individual copies of epiallelic species at single-CpG-site resolution.

Discrimination of Epiallelic Variants Based on Melt Temperature

Bisulfite DNA conversion

Tumor DNA

Bisulfite conversion

PCR

Low GC

High GC

\( T_m \) (°C)

77.4

78.0

78.6

79.2

79.8

80.5

BRCA1
Assessment of BRAC1 Epigenetic Heterogeneity MDS/MPN Patient Samples

A

<table>
<thead>
<tr>
<th>Meth Dens.</th>
<th># Expect</th>
<th># Detect</th>
<th>Avg. Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>75.6</td>
<td>76</td>
<td>77.3</td>
</tr>
<tr>
<td>20%</td>
<td>4.2</td>
<td>3</td>
<td>78</td>
</tr>
<tr>
<td>100%</td>
<td>4.2</td>
<td>5</td>
<td>80.5</td>
</tr>
</tbody>
</table>

B

C

EF: 1.7%

D

EF: 0.05%
Microfluidic Array Chip for Digital Melt Analysis of Methylation Heterogeneity

4 x 4,096 wells

Sample 1
Sample 2
Sample 3
Sample 4

\[
\frac{d\text{RFU}}{dT}
\]

Temperature (°C)

\[
\begin{align*}
70 & \quad 75 & \quad 80 & \quad 85 & \quad 90 & \quad 95 \\
-2 & \quad 0 & \quad 2 & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12
\end{align*}
\]
Integrate DNA Isolation and Bisulfite Conversion Using Silica Superparamagnetic Particles

**Methylation on Beads (MOB)**

- Induced magnetic property in a magnetic field, easy for manipulation
- Reversible DNA binding and desorption by tuning the buffer condition
- Small size provides large surface area for DNA binding
- Size varies from 10 nm to 1000 nm

Comparison of MOB and Conventional Method
Table 1. Clinical information for primary pancreatic samples

<table>
<thead>
<tr>
<th>Pancreas Primary Tumors (N=123)</th>
<th>Normal (N=4)</th>
<th>PanINs (N=20)</th>
<th>Stage I (N=38)</th>
<th>Stage II (N=78)</th>
<th>Stage III (N=5)</th>
<th>Stage IV (N=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Survival (months)</td>
<td>137.3</td>
<td>42.4</td>
<td>17.8</td>
<td>13.9</td>
<td>19.6</td>
<td></td>
</tr>
</tbody>
</table>

(Yi et al., Clinical Cancer Research, 2013)
**Methylation Detection in Sera**

Table 2. Sensitivity and specificity of *BNC1* and *ADAMTS1* in pancreatic cancer patient serum samples

<table>
<thead>
<tr>
<th>Pancreatic Cancer</th>
<th>BNC1</th>
<th>ADAMTS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>90% (9/10)</td>
</tr>
<tr>
<td>II-IV</td>
<td>32</td>
<td>75% (24/32)</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>79% (33/42)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal</th>
<th></th>
<th></th>
<th>Specificity (%)</th>
<th>Estimated Value</th>
<th>95% CI</th>
<th>Estimated Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26</td>
<td>89%</td>
<td>76-100%</td>
<td>92%</td>
<td>82-100%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CI - confidence interval

(Yi et al., *Clinical Cancer Research*, 2013)
Fully Integrated Device for Robust Methylation Detection

Methylation on Beads (MOB)

Step 1: DNA extraction

- Protease K
- SSB & buffers
- Wash

Step 2: Bisulfite conversion

- Sodium Bisulfite Mixture
- Desulphonation Buffer
- Wash

PCR Detection

Methylation on a Chip
Using **Silica Superparamagnetic Particles (SSP)** as a solid phase within droplets.

Centralized & manual tube based PCR detection

Sample In → Lysis → DNA Binding → Wash → Elution (DNA) → PCR cycling & detection → Answer Out

(Droplet magnetofluidics for integrated DNA preparation and PCR)

b) Movement path of SSP plug

- Surface elevation
- Slit
- Washing buffer 2a
- Washing buffer 2b
- Reaction buffer
- Mini tank filled with mineral oil
- Washing buffer 1
- Sample
- SSP plug
- Lysis/binding buffer
- Reaction basin

Fully Integrated Sample Processing and PCR
Quantitative Detection of Rsf1 marker in droplets
DNA bisulfite Conversion Chip for Methylation Analysis

Sulphonation & hydrolytic deamination
(Bisulfite reagent)

\[
\begin{align*}
\text{cytosine} & \xrightarrow{\text{HSO}_3^-} \text{cytosine sulphonate} \\
& \xrightarrow{\text{H}_2\text{O}} \text{uracil sulphonate} \\
& \xrightarrow{\text{NH}_4^+} \text{uracil}
\end{align*}
\]

Alkali desulphonation
(Desulphonation reagent)

\[
\begin{align*}
\text{uracil sulphonate} & \xrightarrow{\text{OH}^-} \text{uracil}
\end{align*}
\]

A. Stark et al, *Biomedical Microdevices*, 2016
Electromagnetic Droplet Manipulation

Magnetic force:

$$F_m = N_{mp} \frac{V_{mp} \chi_{mp}}{\mu_0} B_0 \frac{\partial B_z}{\partial x}$$
**Mechanism of Mixing**

- **Background field**
  - Upward field for bead attraction
  - Downward field for bead repulsion
  - External magnetic field

- **Coil-generated field**
  - Parallel
  - Anti-parallel

- **Graph**
  - $B_z$ (mT) vs $x$ (mm)
  - Solid line: Upward field for bead attraction
  - Dashed line: Downward field for bead repulsion
  - External magnetic field

**Images**
- **Attraction**
- **Repulsion**
- **Release**
On-Chip DNA Extraction and Real-time PCR
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Stand Up to Cancer

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