Oral Cancer Screening Based on Methylation Frequency Detection in hTERT Gene Using Electrochemical Hybridization Assay via a Multi-electrode Chip Coupled with Ferrocenylnaphthalene Diimide

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Oral cancer screening based on methylation frequency detection in *hTERT* gene using electrochemical hybridization assay via a multi–electrode chip coupled with ferrocenylnaphthalene diimide

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Abstract: Ferrocenylnaphthalene diimide-based electrochemical hybridization assay via a multi-electrode chip was applied to detect the methylation frequency in the promoter region of *human telomerase reverse transcriptase* (*hTERT*) gene for clinical samples from tissues, local exfoliated oral cells from a lesion, or from entire oral cavity after their methylation specific PCRs. These methylation frequencies were increased with cancer progress as the following order: healthy volunteers, oral leukoplakia as precancerous lesion, and oral squamous cell carcinoma (OSCC). Operating characteristic analysis of the obtained current data doesn't only give excellent discrimination ability of OSCC, but also of oral leukoplakia from healthy volunteers for all samples. Sensitivity and specificity was 95 % and 90 %, respectively, which is a comparable with methods in practical use.

Keywords: *Human telomerase reverse transcriptase* (*hTERT*) gene • aberrant methylation • oral cancer screening

1. Introduction

Oral cancer diagnosis under early stage is not only very important from standpoint of maintenance of normal oral function, but also extending health span. Until now, cytological diagnosis, where cells are collected by the scraping by interdental brush, sponge brush, or swab and evaluate the presence of atypical cells, is carried out and subsequently confirmed diagnosis was achieved with histopathological manifestation from microscopic appearance of incisional biopsy after Hematoxylin-Eosin staining when precancerous lesion or malignant tumor is suspected.

On the other hand, telomerase is the specialized ribonucleoprotein complex that caps chromosomal ends and stabilizes telomeres by adding "TTAGGG" repeats to the end of chromosomes. Since telomerase activity express in most human cancers but usually not in normal somatic cells [1], telomerase has been focused as diagnostic marker for the purpose of the early detection of human cancers [2]. We have been developing an electrochemical telomerase assay as an indicator of telomerase activity to diagnose oral cancer under early stage [3, 4]. *Human telomerase reverse transcriptase* (*hTERT*) is a major component of telomerase and it is known that *hTERT* expression has been detected in about 85-95% of human malignant tissues [6], and epigenetic hypermethylation in the CpG-rich sequences in the increases during human carcinogenesis [5]. *hTERT* Promoter regions of *hTERT* gene plays an important role in the regulation of *hTERT* expression [7]. Our search of the literature revealed no reports which analyze the methylation status in the promoter region in *hTERT* gene using exfoliated oral cells sample. On the other hand, hyper methylation of CpG sites in promoter region of *hTERT* gene, which is corded gene of active site of telomerase, observed in stomach or bowel cancer and is expected as cancer marker [8].

Several methods for the analysis of methylation status have been reported; methylation-specific PCR (MSP), bisulfite genomic sequencing, real time MSP, MethyLight, combined bisulfate restriction analysis (COBRA) and pyrosequencing [9]. These methods have some disadvantages such as complicated operations and high-cost

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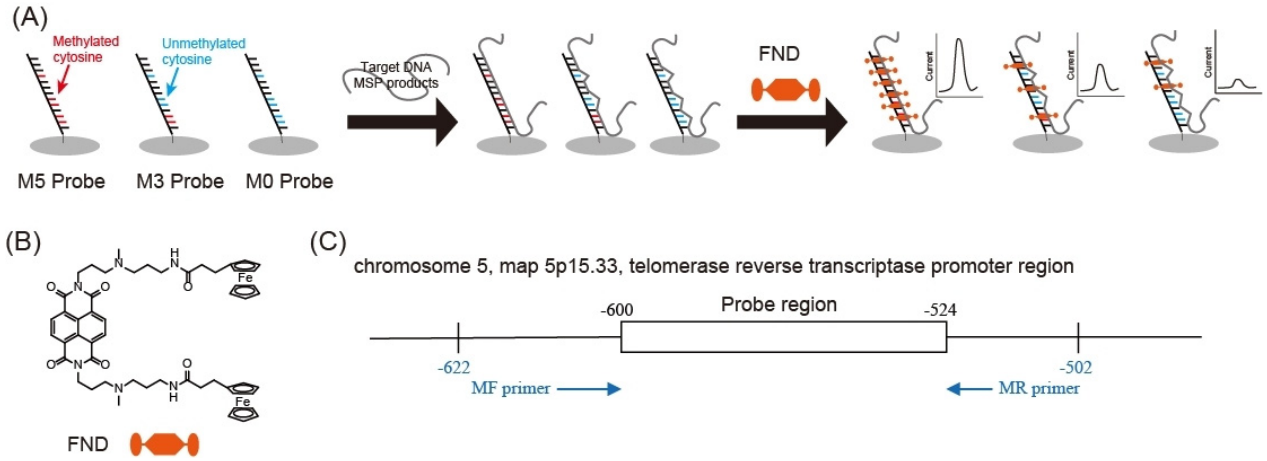

Fig. 1. (A) Strategy of electrochemical hybridization assay (EHA), (B) structure of FND, (C) map of *hTERT* promoter region showing probe region and primers used for MSP (MF primer and MR primer).

running. To overcome such disadvantage, we reported methylated DNA detection method using the ferrocenylnaphthalene diimide (FND)-based electrochemical hybridization assay (EHA) as shown in Figure 1 A. This simple technique is based on fact that FND (Figure 1B) is concentrated double-stranded DNA specifically by threading intercalation [10]. We applied the electrochemical hybridization assay for PCR products from synthetic oligonucleotide, genome DNA from one oral squamous cell carcinoma (OSCC) patient, and one healthy volunteer as template using multi-electrode chip to detect methylation frequency of CpG sites of *hTERT* promoter regions [11]. The obtained results showed that methylation frequency of sample DNAs was realized by the electrochemical hybridization assay with DNA probes carrying different amounts of methylation on the chip and also showed the good correlation between cancer development and methylation frequency [11]. This shows the usefulness of this method as an oral cancer diagnosis.

Here, methylation frequency of the promoter region in *hTERT* gene was searched using for 35 patients of oral squamous cell carcinoma (OSCC) in addition to 25 oral leukoplakia patients, or 34 healthy volunteers, and statistical analysis was performed from these results. Oral leukoplakia is one of precancerous lesion and its diagnosis is necessary because of its malignant transformation rate of 4–20 % [12]. Furthermore, the results from the electrochemical DNA chip was immunohistochemical compared with the expression level of *hTERT* protein. Especially, samples used in this work were collected by the scraping by sponge brush assuming (EOC-E) as a self-diagnosis system by the patient's own or by the scraping by interdental brush assuming (EOC-L) as diagnostic system by dentists in general dental hospital.

2. Experimental

2.1 Materials

FND was synthesized by the procedure previously reported [12]. Oligonucleotides were custom synthesized by Genenet (Fukuoka, Japan). The sequences of probe DNA which are disulfide derivatives are shown in Table 1. PCR primers were as follows: MF primer; 5'-GAG GTA TTT CGG GAG GTT TCG C-3', MR primer; 5'-ACT CCG AAC ACC ACG AAT ACC G-3'. RNase-free water, Formamide (Deionized), 20 × SSC (0.30 M Sodium citrate, 3.0 M NaCl, pH 7.0), 2.0 M potassium acetate (AcOH-AcOK, pH 5.5), 3.0 M KCl and 5.0 M NaCl were purchased from Life technologies (Carlsbad, CA, USA). L(+)-Ascorbic acid was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). DNeasy Blood and Tissue Kit, BisulFlash DNA Modification Kit and ZymoTaq Premix were purchased from Qiagen GmbH (Hilden, Germany), EpiGentek (Farmingdale NY, USA), and Zymo Research (Orange, CA, USA).

2.2 Clinical Samples

This research was approved by the ethics committee of Kyushu Dental University, Kitakyushu, Japan (Approval Number 14-45). Clinical samples (Table 2) were collected from OSCC, oral leukoplakia, and healthy volunteers after obtaining informed consent. Procedure of sample treatment was detailed in supporting materials. Clinical specimens were tissue, local exfoliated oral cells (EOC-E) and exfoliated oral cells from entire oral cavity (EOC-L). Methylation specific PCR (MSP) products from clinical samples were treated by using DNeasy Blood and Tissue Kit, BisulFlash DNA Modification Kit and PCR with ZymoTaq Premix according to a procedure described previously [11].

Table 1. Probe DNA sequences immobilized on the electrodes.

Probe DNA	Sequence
M5(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TCG CGT TTA CGC GTT TTC GTT-3′
M4(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TCG CGT TTA CGC GTT TTT GTT-3′
M4′(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TTG CGT TTA CGC GTT TTC GTT-3′
M3(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TCG CGT TTA CGT GTT TTT GTT-3′
M3′(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TTG TGT TTA CGC GTT TTC GTT-3′
M2(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TCG CGT TTA TGT GTT TTT GTT-3′
M2′(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TTG TGT TTA TGC GTT TTC GTT-3′
M1(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TCG TGT TTA TGT GTT TTT GTT-3′
M1′(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TTG TGT TTA TGT GTT TTC GTT-3′
M0(+)	5′-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-TAG TTG TGT TTA TGT GTT TTT GTT-3′

Table 2. Number of clinical samples

	n Tissue	EOC-L	EOC-E
OSCC	35	22	21
oral leukoplakia	25	24	20
healthy volunteers	34	23	29

2.3 Electrochemical Hybridization Assay (EHA)

Custom-made multi-electrode chip (Tanaka Kikinzoku Kogyo K. K., Tokyo, Japan), carrying 10 working electrodes, one counter electrode, and one reference electrode, was used as a sensor chip (Figure S1).

Probe DNAs (Table 1) were immobilized to a multi-electrode chip according to the pattern of probe DNA by the previously reported method [11]. Reaction mixture, 0.25 ng/μL MSP products, 5 × SSC and 50% Formamide (Deionized)) was incubated for 15 minutes at 95°C, after that, it was stored at 4 °C until use. Hybridization with the reaction mixture was carried out by soaking a probe DNA-immobilized electrode in 4.0 μL of reaction mixture for 2 h at 20 °C. Differential Pulse Voltammetry (DPV) was measured in 0.20 M AcOK-AcOH, 0.20 M KCl, 5.0 μM FND and 10 mM L(+)-ascorbic acid solution by using IM-AN100 before and after hybridization (Figure 1). The following parameters for DPV measurement were employed“; Initial Voltage = -0.1 V; final Voltage = 0.3 V; Increasing Voltage = 10 mV; amplitude = 0.05 V; pulse width = 0.025 s; sample width = 0.005 s; pulse period = 0.01 s; quiet time = 2 s; and resistance = 510 kΩ. All measurement data were standardized using Δi values, which are defined as $(i/i_0\text{-}1) \times 100$ %, where i_0 and i refer to the current value of DPV before and after hybridization, respectively.

2.4 Statistical Analysis

The results of EHA analyzed using by the Student *t* test. All analyses were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Differences with *p* values of less than 0.05 were considered significant.

3. Results and Discussion

3.1 Electrochemical Hybridization Assay

PCR primer containing 24-mer sequence carrying five CpG sites in 121-mer region of *hTERT* promoter (Figure 1C) was selected to be around 58°C of T_m values of its duplex after bisulfate treatment using CpGWare software (http://apps.serologicals.com/CPGWARE/dna_form2.html). DNA probe sequences (M0, M1, M1', M2, M2', M3, M3', M4, M4', and M5) were prepared with ten different methylation frequency containing from one to five methylated sites [10] as shown in Table 1. Clinical samples were amplified with PCR using methylation specific primer and the obtained PCR products were treated with the electrochemical DNA chip. Typical DPV data for OSCC samples were included in Figure S2 as ESI as follows. Figure 2 shows current shift, current shifts in M4, M4', and M5 for higher methylated probes were higher than other probes in the case of OSCC (Figure 2 A). Current shifts for healthy peoples were totally

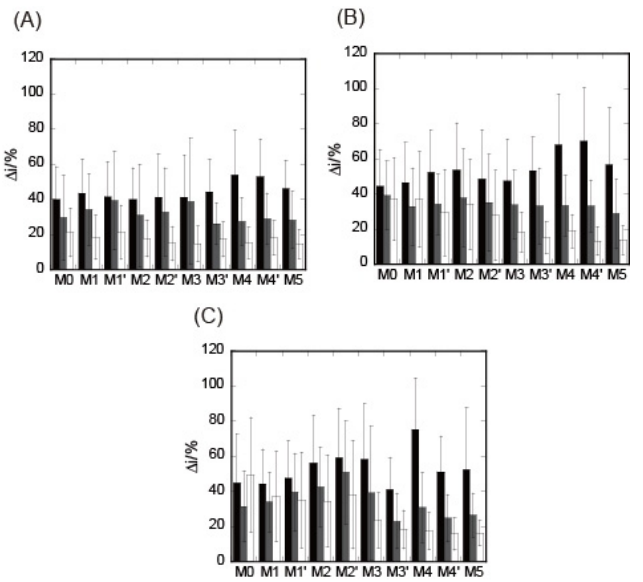

Fig. 2. Current increasing rate of the multi-electrode chip after hybridization with the genomic DNAs from the tissue (A), EOC–L (B) and EOC–E sample (C) from OSCC (Black bar), oral leukoplakia (Gray bar) and healthy volunteers (White bar) treated with bisulfite modification and MSP.

lower values, which are in agreement with data reported previously. In the case of oral leukoplakia as a precancerous lesion, relative larger values shows in the case for 1–3 methylated probes of M1, M1', M2, M2', M3, and M3'. Previous paper [11] reported by us showed the current shifts of 80%, 60%, and 40% for fully matched, single mismatched, and two mismatched hybrids, respectively and current shift increased with increase of target DNA. We applied the electrochemical hybridization assay for PCR products from synthetic oligonucleotide, genome DNA from one oral squamous cell carcinoma (OSCC) patient, and one healthy volunteer as template using multi-electrode chip to detect methylation frequency of CpG sites of *hTERT* promoter regions [11]. In the case of our previously paper [11], synthetic oligonucleotide was used as template of PCR, which contained single sequence. On the other hand, DNA from clinical samples were mixture containing different methylation pattern, which is confirmed by the sequencing of MSP product from genomic sample [10]. This result leads to slightly discrepancy of the current shift between the data in this experiment and in previously paper [11].

According to these behaviours, samples of oral leukoplakia were expected to form mainly two mismatched hybrids with 1–3 methylated probes. When current shifts for M4 and M5 gave moderate ones, it is expected these mismatched hybrids carrying different methylated sites without increasing methylation frequency because of low current shift for M4 and M5. For example, when methylated (M) and unmethylated (U) sites were noted, M3 sequence became to show as 5'-MMMUU-3' and two mismatched is expected to carry same methylation number such as 5'-MUMMU-3', not increased methylation number such as 5'-MMMMM-3' and thus methylation frequency of these samples were expected to be 1/5 – 3/5, or 20–60%. M3' probe also showed same situation with M3. Analysis for oral leukoplakia has been carried out for p16 [14] or TSPYL5 gene [15] using MSP and showed the amplification for unmethylated primer and 30% of amplification for methylated one. However, this report didn't know that amplified DNA by methylated primer doesn't tell 100% methylation or not. Comparing with these results, EHA analysis using 10-different DNA probes after MSP gives methylation frequency of an internal sequence, where we can show methylation frequency as example of patients of oral leukoplakia. Similar behaviour was obtained in EOC–L (Figure 2B) or EOC–E (Figure 2C).

Under OSCC, M4, M4', and M5 as DNA probes carrying higher methylation frequency showed higher current shift than for other DNA probes, however, under healthy volunteers, M0, M1, M1', M2, and M2' as DNA probes carrying lower methylation frequency showed higher current shift than other DNA probes. These results suggested that misannealing is unfavourable for tissue sample because of less contamination of normal cell and that misannealing is favourable for EOC–E because of many contamination of normal cells resulting in the misannealing amplification of unmethylated DNA and subsequently higher current shift for M0, M1, M1', M2, and M2'.

Relative higher current shifts shows for M1, M1', M2, M2', M3, and M3' as DNA probe carrying 1–3 methylated sites for EOC–L (Figure 2B) or EOC–E (Figure 2C) in oral leukoplakia like as tissue sample.

3.2 Establishment of Cancer Diagnosis

Figure 3 shows the Box plots using current shift for M5 probe for the highest methylation frequency. Current shift for oral leukoplakia located middle value between OSCC and healthy volunteers. Statistics analysis using student-t test of these data gave a significant deference with $p < 0.05$. OSCC and healthy volunteers were discriminated whereas oral leukoplakia couldn't discriminate from OSCC, where response of tissue, EOC–L, and EOC–E was overlapped with that of OSCC in this order. Figure 2 shows that current shift of M4 and M4' is larger than that of M5 for OSCC. Since methylation frequency was 80–100% for cancer state, evaluation only using M5 loses signal from data without 100% methylation frequency resulting in scattered data for OSCC. As shown in Table 3, 80% methylated samples shows double or single mismatched, or fully matched DNA duplex with M5, M4, and M4′ probe and thus high methylation samples were evaluated using the sum of the current shift from M5, M4, and M4′ probe ($\Delta i_{M4+M4'+M5}$).

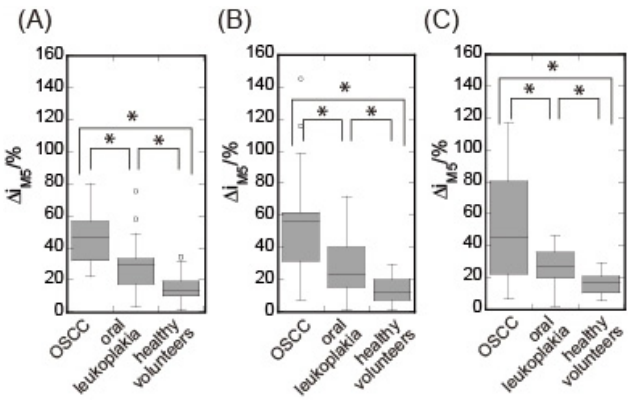

Fig. 3. Box plots of Δi_{M5}/% of EHA in Tissue (A), EOC-L (B) and EOC-E samples (C). *: $p < 0.01$.

Thus Box plots using the $\Delta i_{M4+M4'+M5}$ as shown in Figure 4. Overlap is rarely met between OSCC, oral leukoplakia, and healthy volunteers. In this evaluation, response from OSCC and oral leukoplakia didn't overlap in all samples from tissue, EOC–L, and EOC–E. Discrimination accuracy is improved by this synthetic judgment. ROC analysis was carried out using $\Delta i_{M4+M4'+M5}$ value.

ROC analysis was carried out in the case of the detection of OSCC from all samples (Table 4) and of the detection of OSCC and oral leukoplakia from all samples

Table 3. Combination between probe DNA (M5, M4, M4') and Target DNA

		Probe DNA M5 3'-MMMMM	M4 3'-UMMMM	M4' 3'-MMMMU
Target DNA	5'-MMMMM	FM	1MM	1MM
	5'-UMMMM	1MM	FM	2MM
	5'-MUMMM	1MM	2MM	2MM
	5'-MMUMM	1MM	2MM	2MM
	5'-MMMUM	1MM	2MM	2MM
	5'-MMMMU	1MM	2MM	FM

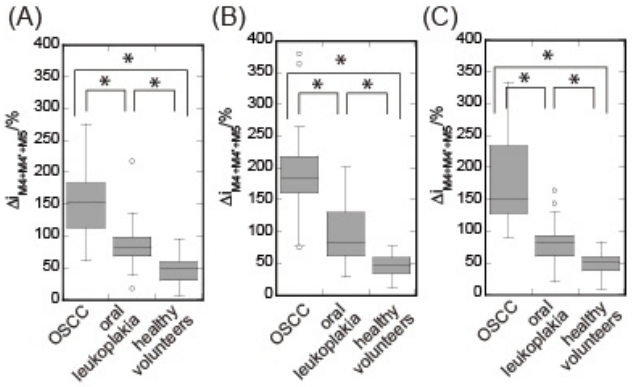

Fig. 4. Box plots of $\Delta i_{M4+M4'+M5}$/% of EHA in Tissue (A), EOC-L (B) and EOC-E samples (C). *: $p < 0.01$.

Table 4. ROC analysis in the case of the detection of OSCC from all samples.

	M5 + M4 + M4'/ OSCC OSCC	oral leukoplakia	healthy volunteers	accuracy rate	AUC
Tissue	30/35	7/25	1/34	86 % (81/94)	0.927
EOC-L	19/22	4/24	0/23	90 % (62/69)	0.936
EOC-E	20/21	5/19	0/29	91 % (63/69)	0.970

AUC: area under the curve, Threshold: Tissue 96.0 %, EOC–L 134 %, EOC–E 96.6 %

(Table 5). Table 4 shows 0.927–0.970 of the area under the curve (AUC) under detection of OSCC resulting in high reliability in this evaluation. Positive diagnosis rates were 86, 90, and 91 % for tissue, EOC–L, and EOC–E, respectively, using the threshold values (Tissue 96.0 %, EOC–L 134 %, and EOC–E 96.6 %). Positive diagnosis rates were 84, 90, and 88 % for tissue, EOC–L, and EOC–E, respectively, under the detection of cancer and precancerous lesion as shown in Table 5, which is similar performance in the cancer detection. This result doesn't only show the usefulness of detection of cancer, but also of precancerous lesion in this system coupled with the selection of the threshold of $\Delta i_{M4+M4'+M5}$.

Table 5. ROC analysis in the case of the detection of OSCC and leukoplakia from all samples.

	M5 + M4 + M4'/ OSCC + OL OSCC	oral leukoplakia	healthy volunteers	accuracy rate	AUC
Tissue	33/35	16/25	4/34	84 % (79/94)	0.906
EOC-L	22/22	18/24	1/23	90 % (62/69)	0.919
EOC-E	21/21	14/19	3/29	88 % (61/69)	0.904

AUC: area under the curve, Threshold: Tissue 74.6 %, EOC–L 71.4 %, EOC–E 63.2 %

4. Conclusions

Methylation frequency in the promoter region of *hTERT* gene was increased with cancer progress as the following order: healthy volunteers, oral leukoplakia, and OSCC. Sensitivity and specificity was 95 % and 90 %, respectively, in this method which is a comparable with methods in practical use (Table 6). When comparing with the specificity, this method is higher than the method using telomerase activity, which is reported previously [3,4]. However, this method takes 2 h, whereas the method using telomerase activity was finished around 30 min.

Table 6. Sensitivity and specificity of cancer diagnosis methods

	Method	Sensitivity/ %	Specificity/ %	Ref.
Colon	CEA (carcinoembryonic antigen)	36	87	16
Colon	PAI-1 (plasminogen activator inhibitor1)	94	84	16
Lung	PET (positron emission tomogram)	70	84	17
Breast	Mammography	84	91	18
Oral	TRAP assay	14	59	3
Oral	Electrochemical Telomerase assay	93	86	3
Oral	EHA (This paper)	95	90	

This result shows that methylation frequency in promoter region of *hTERT* gene increases with cancer

development as obtained the good marker of oral cancer diagnosis under early stage. Furthermore, this method should open diagnosis system by the patient's own or by dentist in general dental hospital.

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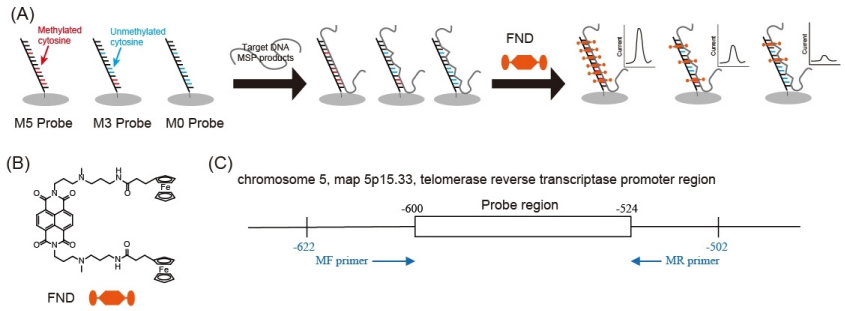

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