バイオ分析化学特論（１３）

酵素的&非酵素的電気化学DNA分析法

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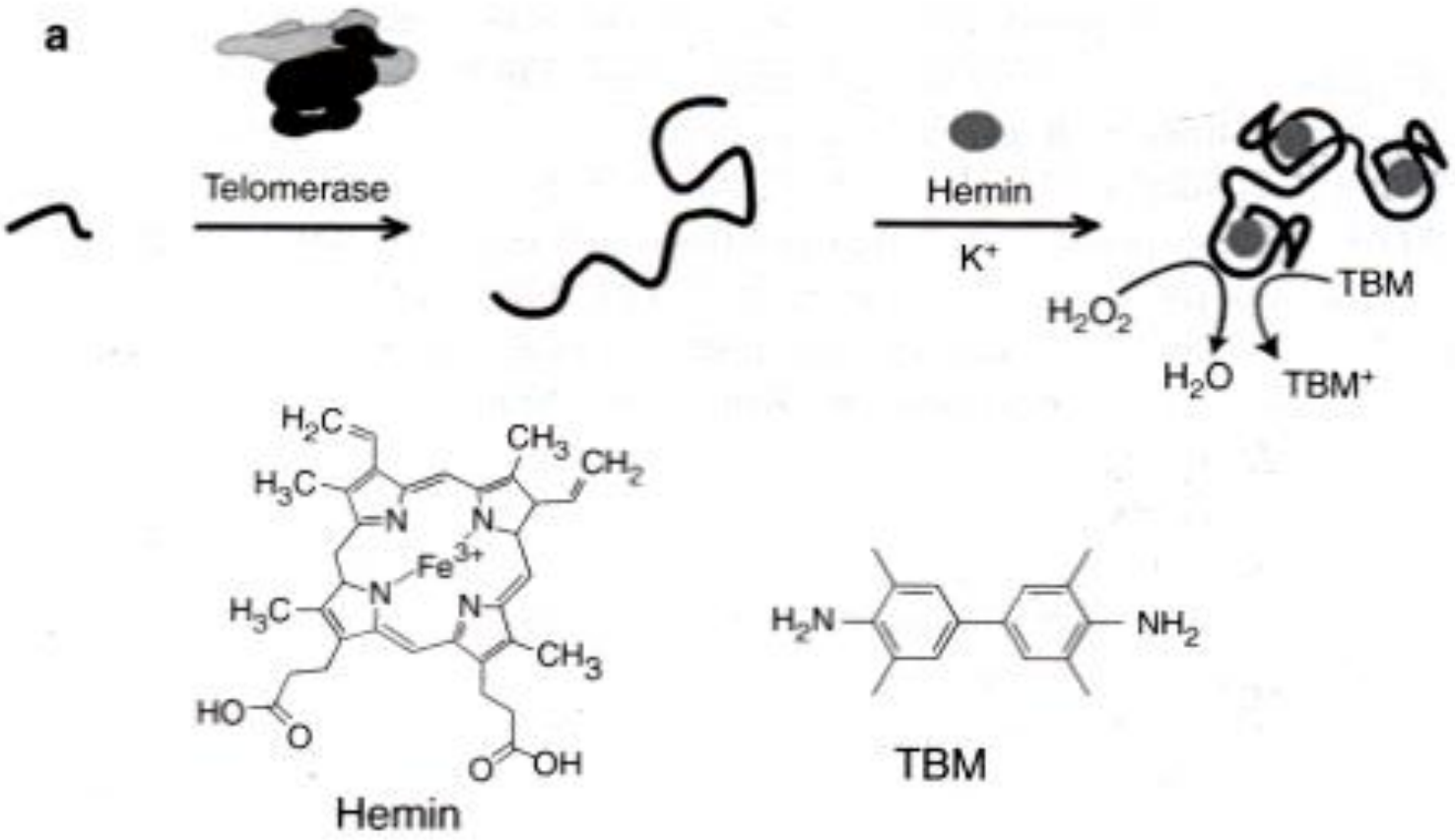
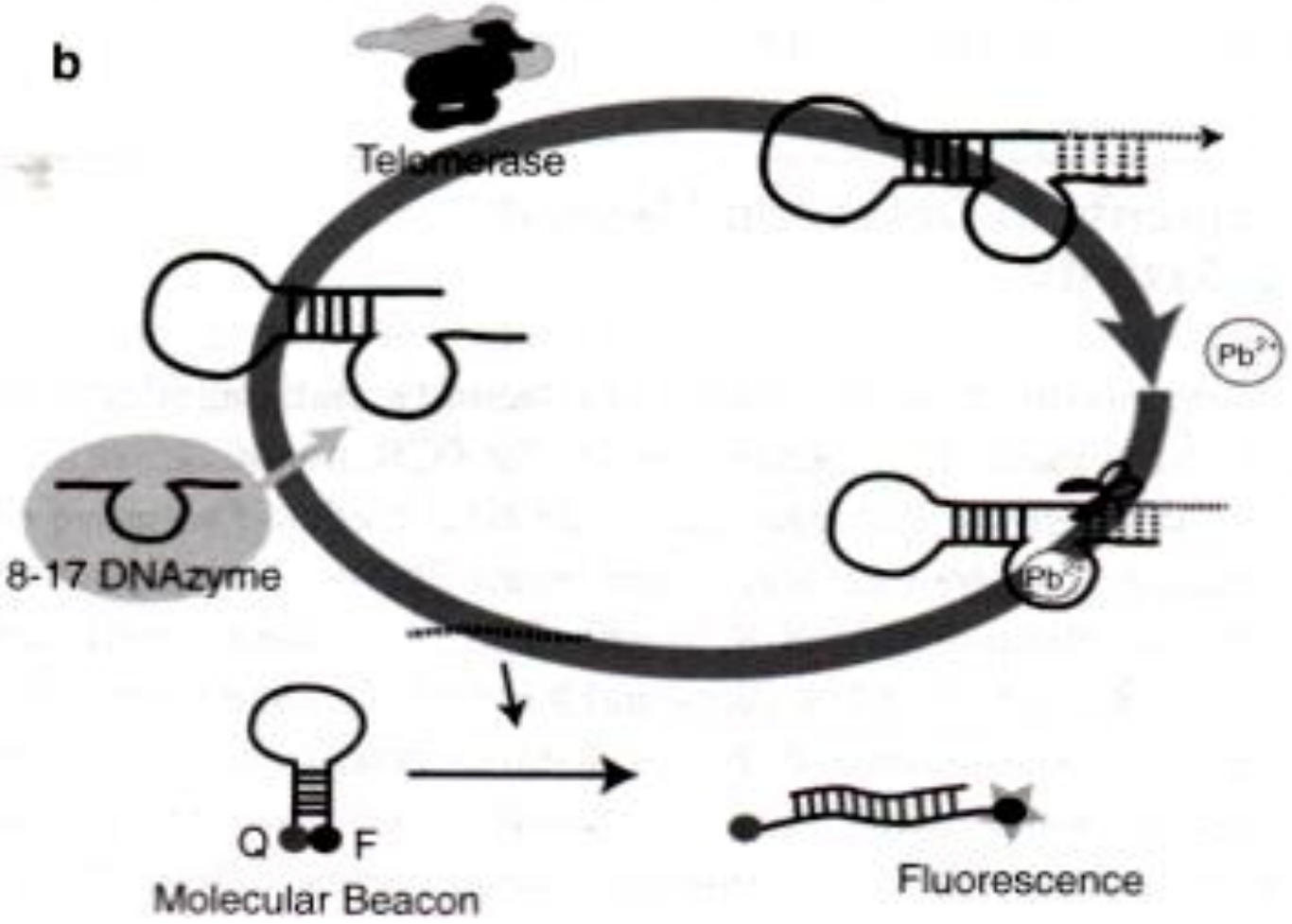

a
Telomerase
Hemin
K+
H2O2
H2O
TBM
TBM+
H2C
CH3
CH2
H3C
N
N
Fe3+
N
N
H3C
CH3
HO
O
O
OH
Hemin
H2N
NH2
TBM


b
Telomerase
8-17 DNAzyme
Pb2+
Pb2+
Q
F
Molecular Beacon
Fluorescence

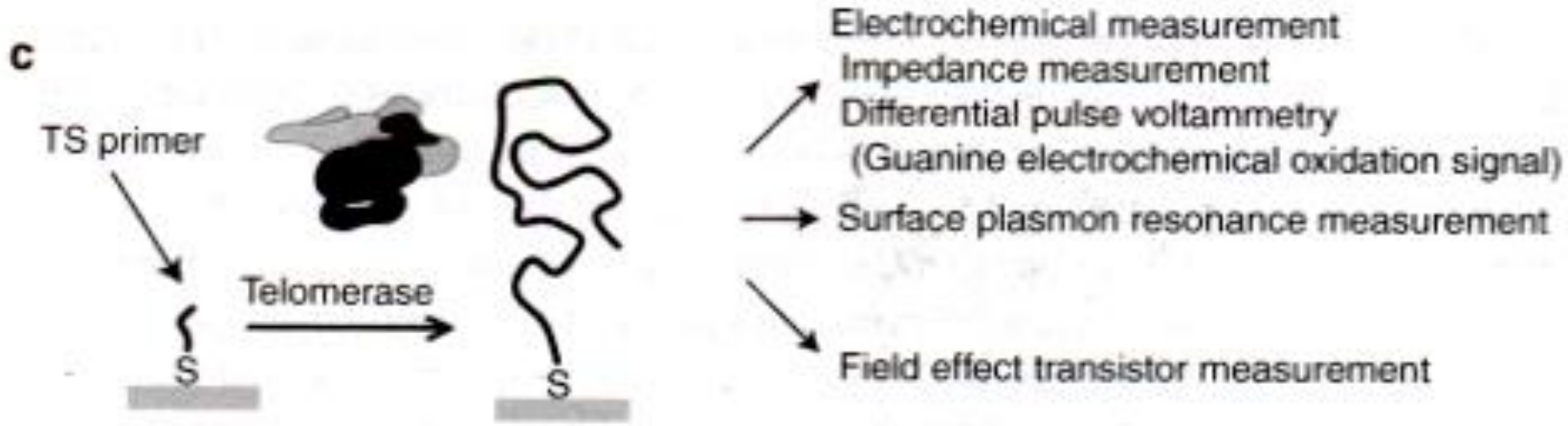
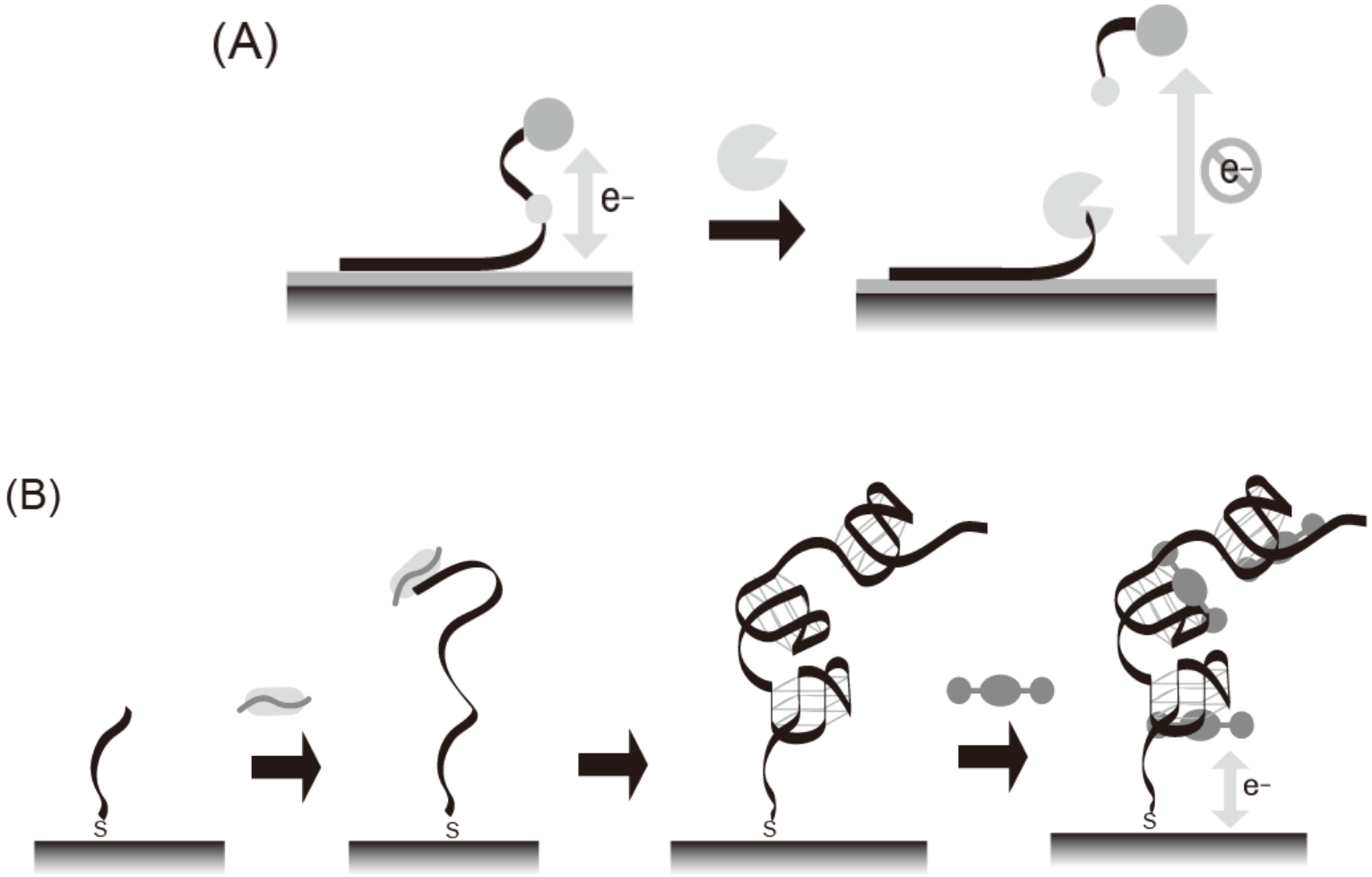


Fig. 2 Principle of non-PCR-based telomerase detection methods. (a) Hemin-catalyzed telomerase detection. The elongated telomerase substrate (*TS*) primer forms a tetraplex structure with bound hemin, and this complex acts as DNAzyme to generate TMB+ from TMB and H_2O_2. Telomerase activity is estimated by measuring the absorbance of TMB^+ at 650 nm.



（A）フェロセン化オリゴヌクレオチド修飾電極を利用したヌクレアーゼ検出の概念．（B）電極上でのテロメラーゼ伸長反応と4本鎖DNA形成．

Triggered amplification by hybridization chain reaction

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Communicated by Stephen L. Mayo, California Institute of Technology, Pasadena, CA, September 24, 2004 (received for review July 2, 2004)

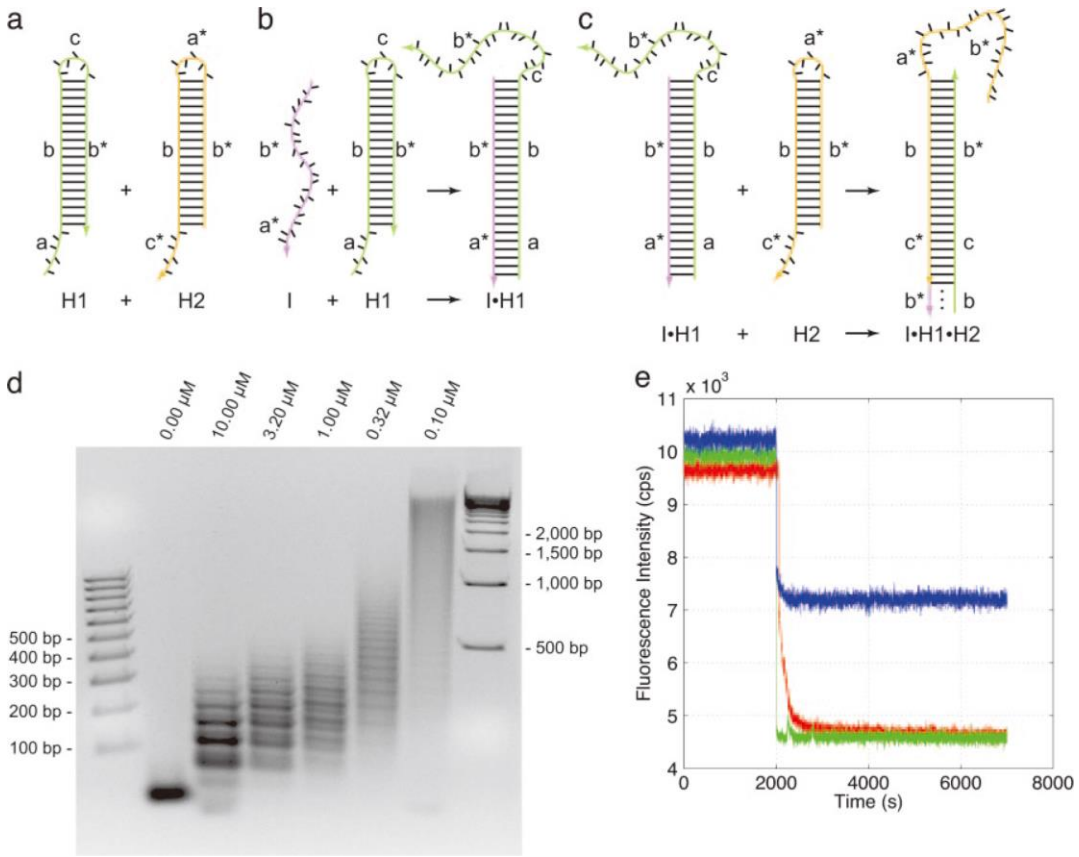

Fig. 1. Basic HCR system. (*a–c*) Secondary structure schematic of HCR function. Letters marked with * are complementary to the corresponding unmarked letter. (*a*) Hairpins H1 and H2 are stable in the absence of initiator I. (*b*) I nucleates at the sticky end of H1 and undergoes an unbiased strand displacement interaction to open the hairpin. (*c*) The newly exposed sticky end of H1 nucleates at the sticky end of H2 and opens the hairpin to expose a sticky end on H2 that is identical in sequence to I. Hence, each copy of I can propagate a chain reaction of hybridization events between alternating H1 and H2 hairpins to form a nicked double-helix, amplifying the signal of initiator binding. (*d*) Effect of initiator concentration on HCR amplification. Lanes 2–7: six different concentrations of initiator (0.00, 10.00, 3.20, 1.00, 0.32, and 0.10 μM) in a 1 μM mixture of H1 and H2. Lanes 1 and 8: DNA markers with 100-bp and 500-bp increments, respectively. (*e*) HCR kinetics. The hairpin monomers do not hybridize before triggering by initiator [($H1^{2AP}$ + 1.2× H2) + 0.5× I, red]. The same quenched baseline is achieved without HCR by adding excess initiator to $H1^{2AP}$ in the absence of H2 ($H1^{2AP}$ + 4.0× I, green). Addition of insufficient initiator to $H1^{2AP}$ provides only partial quenching ($H1^{2AP}$ + 0.5× I, blue).

PNAS, 101, 15275 (2004).

Electrochemical Interrogation of Kinetically-Controlled Dendritic DNA/PNA Assembly for Immobilization-Free and Enzyme-Free Nucleic Acids Sensing

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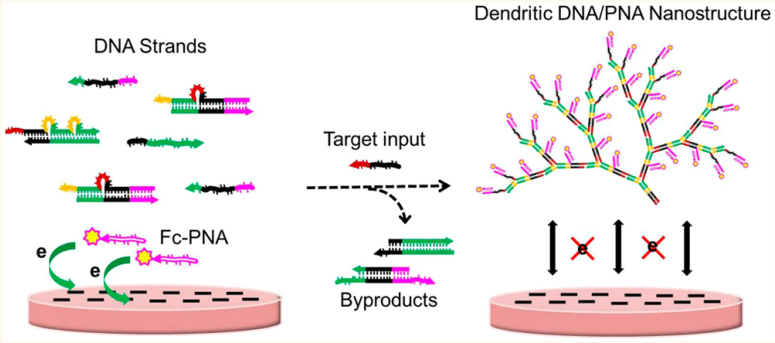
[†]Division of Biomedical Engineering and [‡]Department of Chemical and Biomolecular Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

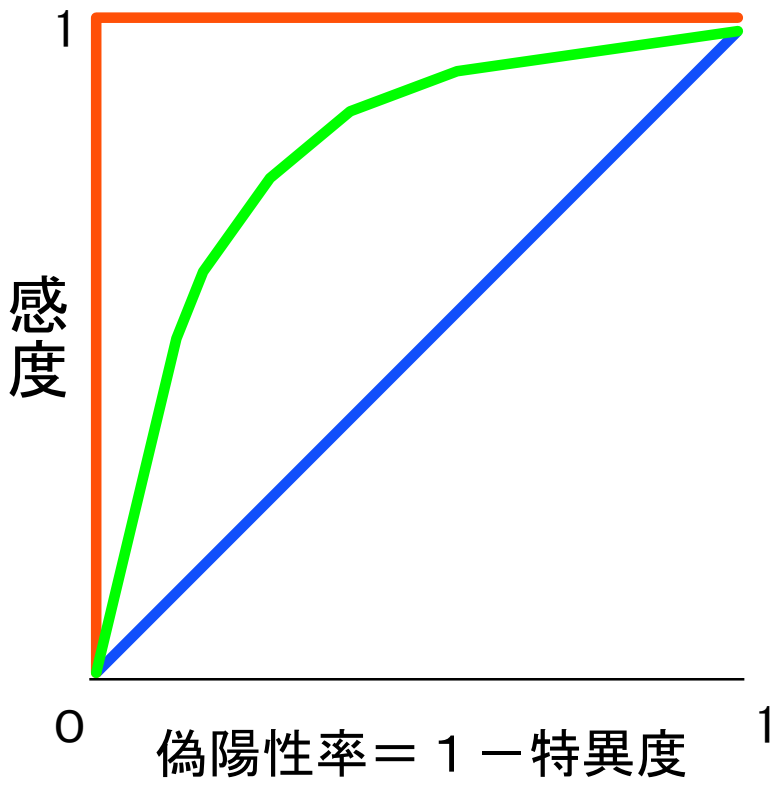
ABSTRACT We present an immobilization-free and enzyme-free electrochemical nucleic acid sensing strategy, which uses kinetically controlled dendritic assembly of DNA and peptide nucleic acid (PNA). In the presence of a target sequence, ferrocene-labeled PNA probes (Fc-PNAs) and specially designed DNA strands are autonomously assembled into dendritic nanostructures through a cascade of toehold-mediated strand displacement reactions. The consumption of freely diffusible Fc-PNAs (neutrally charged), due to incorporation to DNA/PNA dendrimer, results in a significant electrochemical signal reduction of Fc on a negatively charged electrode from which the hyperbranched and negatively charged dendrimer of DNA/PNA would be electrostatically repelled. The cascade-like assembly process and large electrostatic affinity difference between Fc-PNAs and DNA/PNA dendrimer toward the sensing electrode offer a detection limit down to 100 fM and an inherently high specificity for detecting single nucleotide polymorphisms. The target-triggered mechanism was examined by PAGE analysis, and morphologies of the assembled dendrimers were verified by AFM imaging.



KEYWORDS: DNA/PNA dendrimer · kinetically controlled assembly · electrochemical detection · immobilization-free · enzyme-free

ACS NANO, 9, 5027 (2015).

ROC (Receiver Operating Characteristic)解析



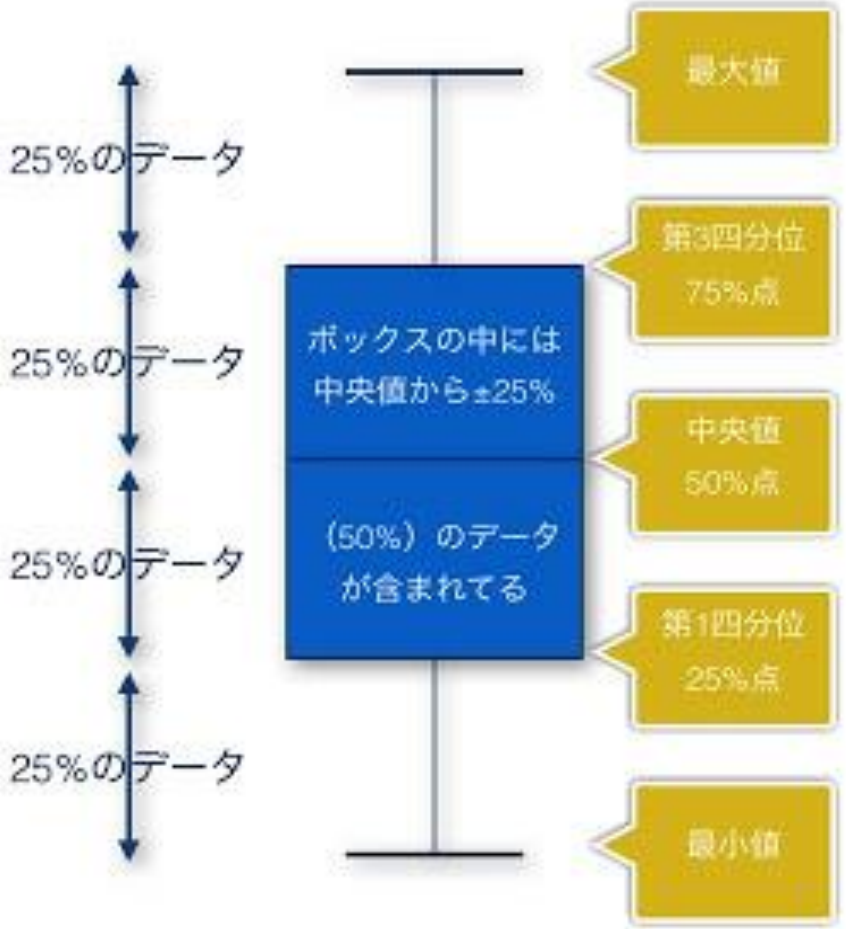
理想的な検査

一般の検査

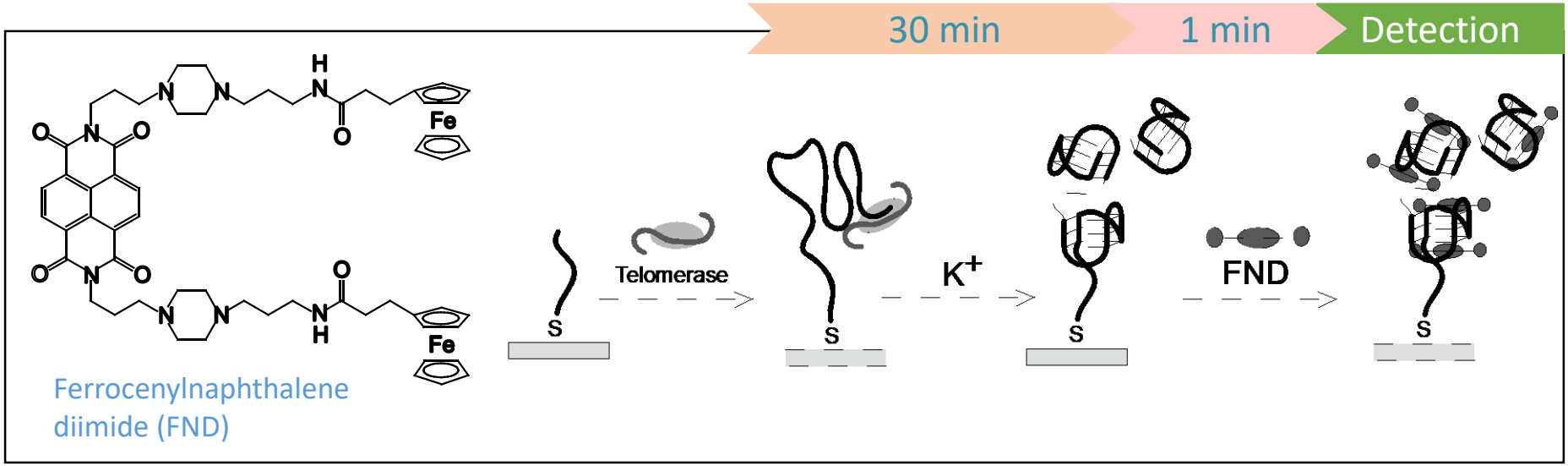
役に立たない検査

曲線が左上に偏っていて
曲線下の面積が大きい検査
ほど有用！

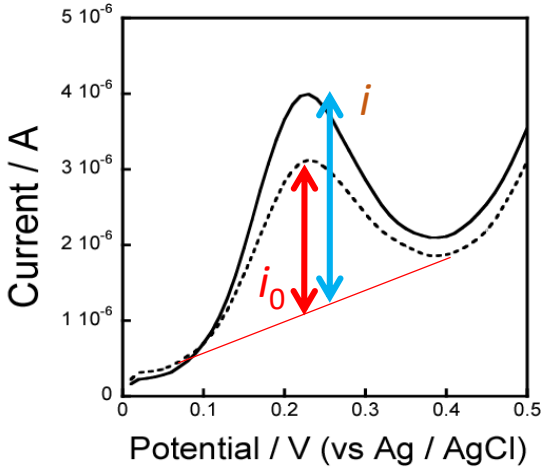
ボックスプロットの見方



Ferrocenylnaphthalene diimide (FND)-based electrochemical telomerase assay (ECTA)



Data analysis



Δi: Current increase ratio
I : Before reaction
i_0 : After reaction

$$\Delta i\ (\%) = (i - i_0)/i_0 \times 100$$

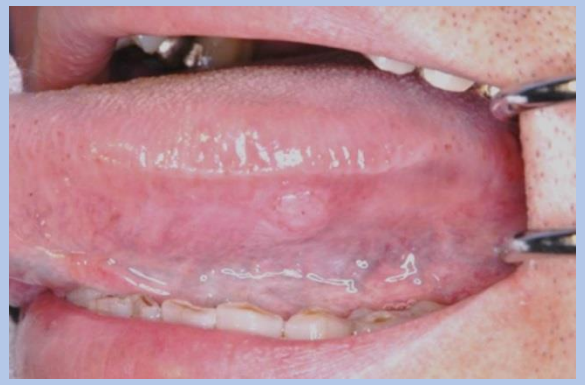
This assay enabled simple and quick analysis without PCR and gel electrophoresis

We applied this assay to tongue cancer diagnosis

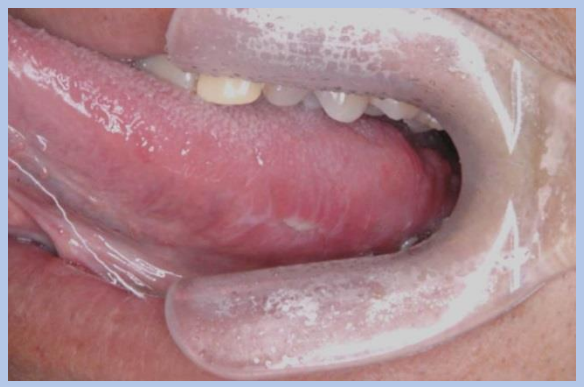
K. Mori *et al.*, *Clin. Chem.*, **59**, 289 (2013).

Importance of early diagnosis of oral cancer

Initial oral cancer



before surgery



After surgery (resection alone)

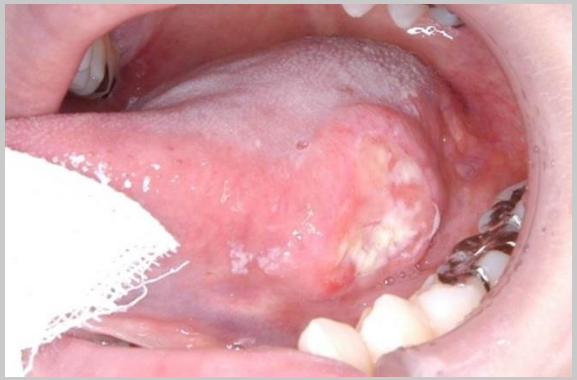
hospital charges: ca. 500,000 yen
(surgical cost: ca. 180,000 yen)
length of hospital stay: ca. 10 days

90%

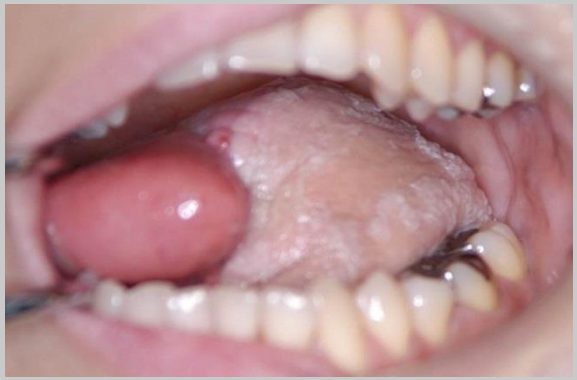
Five year survival rate

Advancing oral cancer

30%



before surgery



After surgery (resection + repair operation)

hospital charges: ca. 3,000,000 yen
(surgical cost: ca. 2,000,000 yen)
length of hospital stay: over1.5 months

QOL decay

Present state of oral cancer in Japan

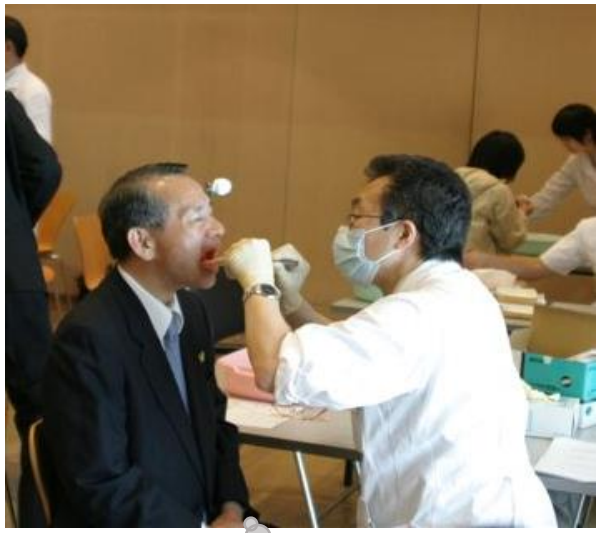
① Increasing patient under super-aging society

② Early diagnosis required medical specialist

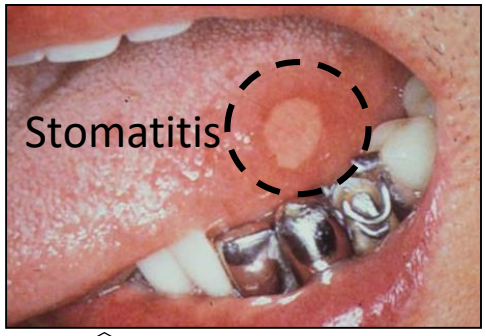
③ Limited number of medical specialist (Doctor of dental surgery)

It is very important to develop early oral cancer diagnosis.

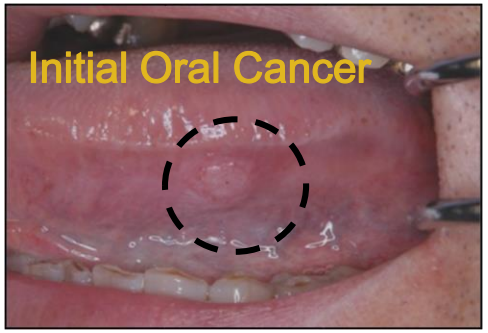
Present oral caner diagnosis

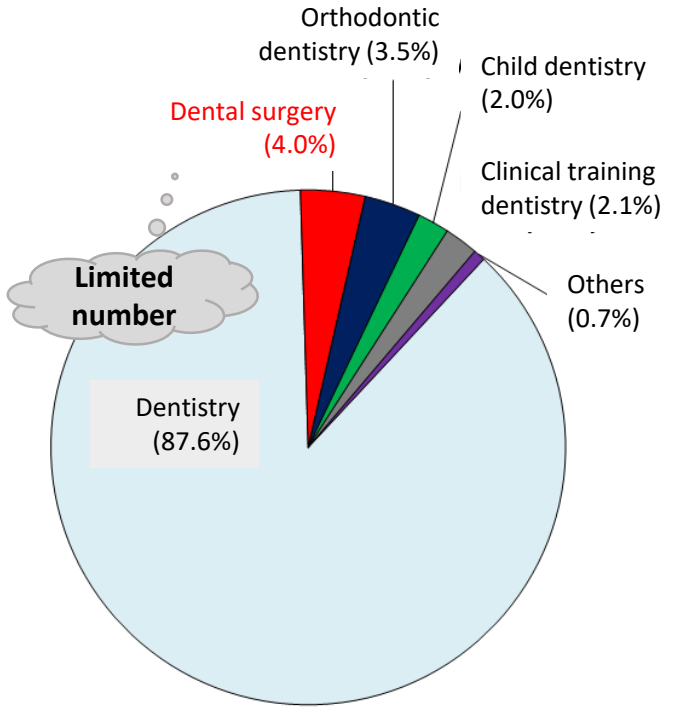


Inefficient method



Hard to discriminate





(出所) 厚生労働省：統計情報・白書（2010）

TERT expression in oral cancer cell lines

oral cancer cell lines

- HSC-2 cells, human oral squamous cell carcinoma cell lines
- HSC-3 cells, human tongue squamous cell carcinoma cell lines
- Ca9-22 cells, human gingival squamous cell carcinoma cell lines
- SAS cells, and human tongue squamous cell carcinoma cells

EXPERIMENTAL

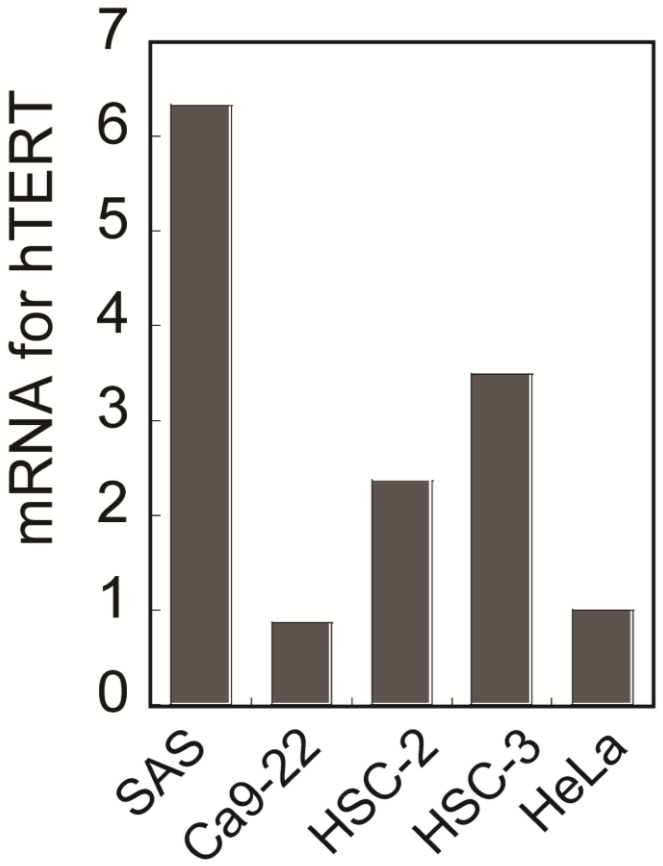
1) Dissolve samples in 500 µL of lysis buffer, centrifuge.

2) Obtain supernatant of cells.

3) Reverse transcription by Super-Script Reverse TranscriptaseII (Invitrogen)

4) RT-PCR by FAST SYBR® Green Master Mix

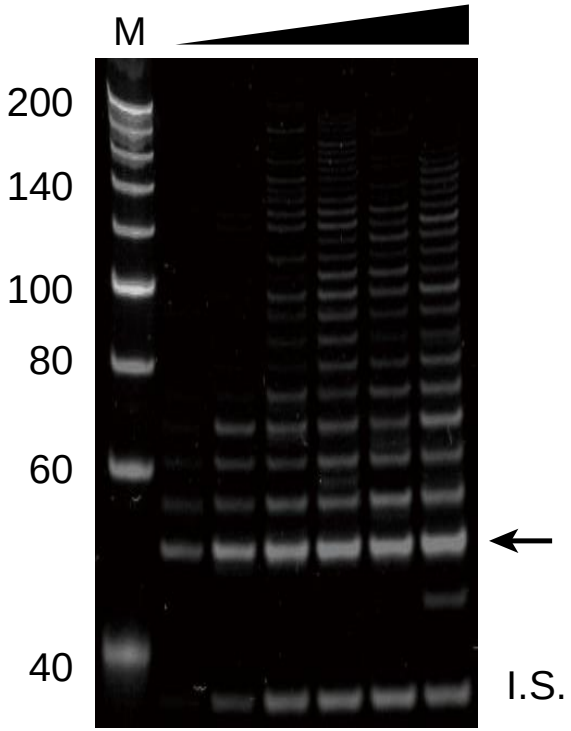
∗ Internal control: GAPDH gene



Higher and lower mRNA concentration for the hTERT, the catalytic subunit gene of telomerase, is SAS and Ca9-22 cells.

TRAP assay in oral cancer cell lines

SAS



Number of cells	SAS	Ca9-22	HSC-2	HSC-3
0	-	-	-	-
10	-	-	-	-
50	＋	-	-	-
75	＋	±	±	±
100	＋	±	＋	＋
200	＋	＋	＋	＋

TRAP Assay Reaction solution(TRAPeze by Millipore)
20 mM Tris-HCl, pH8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 μM each dNTP mixture, 1×TS Primer, 1 ×Primer Mix, 2 Units Taq polymerase,
0, 10, 50, 75, 200 cells
Telomerase reaction, 37 °C, 60 min

ladder=0, -; 0<ladder<5, ±; ladder≧5, +

Detection limit is over 200 cells

ECTA in oral cancer cell lines

-S-5'-TTTTTTTTTT TS primer sequenece AATCCGTCGAGCAGAGTTAGGG

1 min

2 h

1 min

30 min

1 min

Pretreatment of gold electrode: plasma treatment for 30 sec

↓

5 nM SS-T8TS1 in 1 mM MCH, 0.1 M NaCl 300 μL, 37 °C, 2 h

↓

i_0 measurement (SWV)

0.1 M AcOH-AcOK, 0.1 M KCl (pH 5.5), 20 μM FND

↓

Reaction with cell extract, sample solution 20 μL

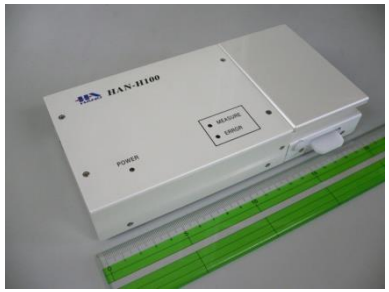
[10 ng/μL, 50 mM Tris-HCl (pH 8.0), 1.0 mM $MgCl_2$, 50 mM KCl, 0.10 mM 2-mercaptoethanol,

0.10 M spermidine, 20 μM dNTP mixture]

37 °C, 30 min

↓

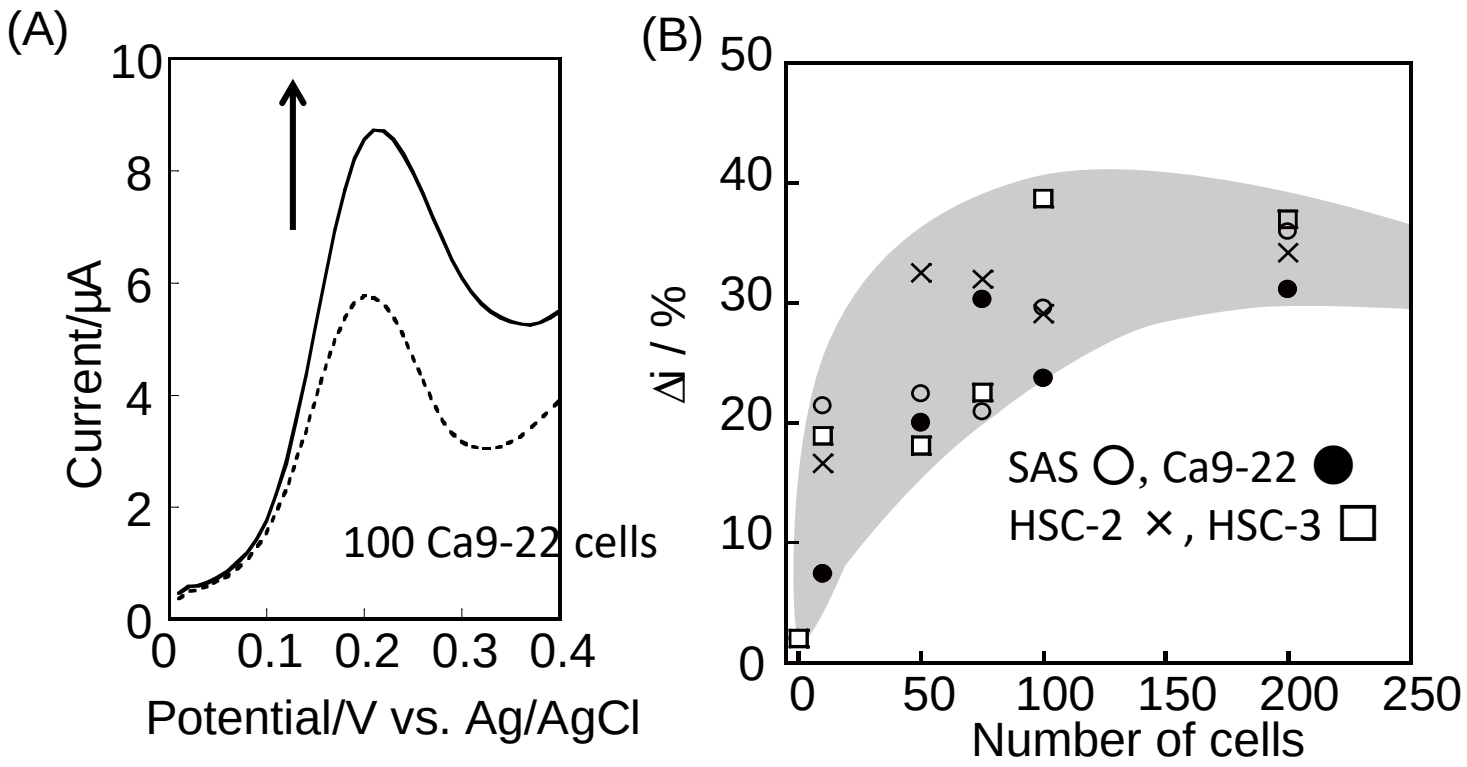
i measurement (SWV)



$$\Delta i\ (\%) = 100 \times (i - i_0)/\ i_0$$

SWV: square wave voltammetry

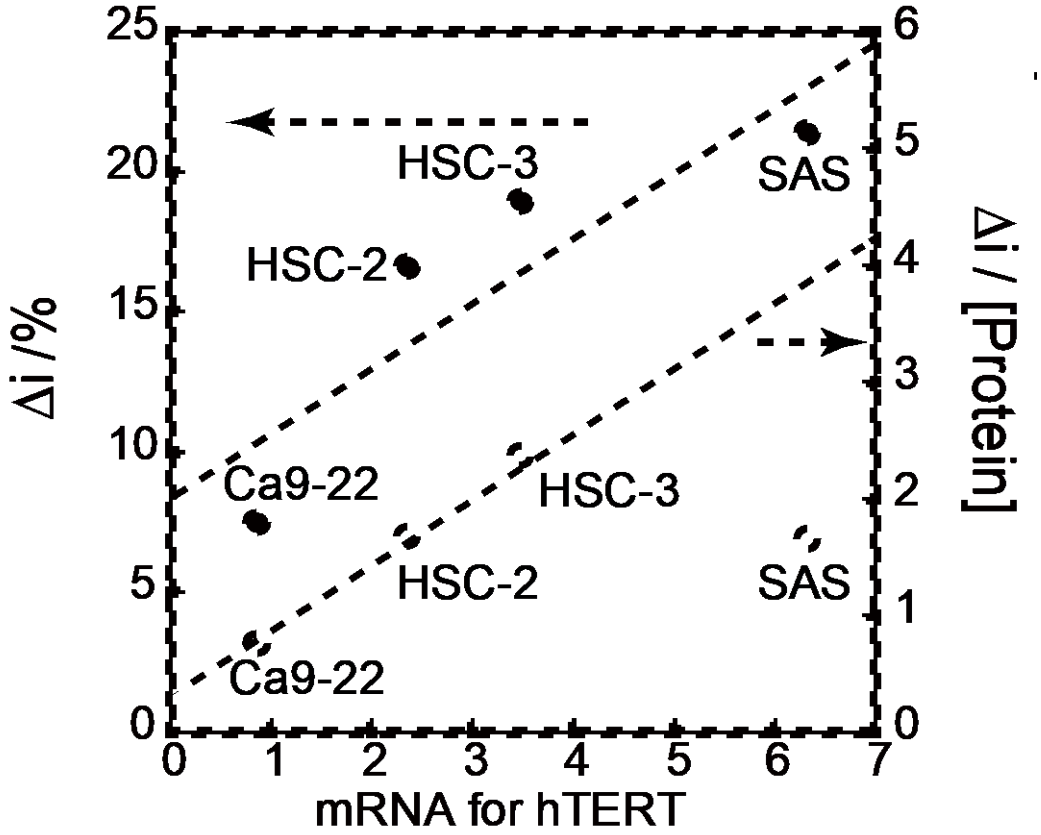
ECTA in oral cancer cell lines



✓**Detection limit is 10 cells** (P<0.001).

✓The detection limit of ECTA was five times lower than that of TRAP.

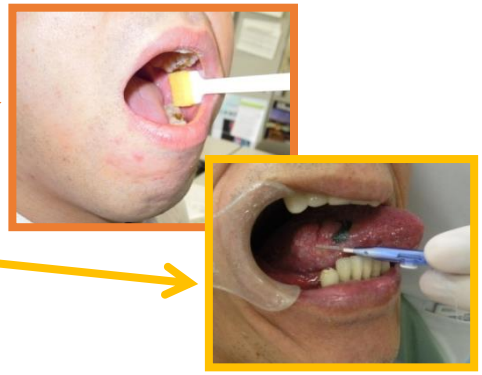
Correlation of the current increase in ECTA with mRNA for correlation



The current increase was found to be proportional to the amount of mRNA for hTERT in the 10-cell lysate quantified by RT-PCR.

Preparation of clinical samples

Medical checkup



Samples

exfoliated buccal cells (B)・・・total oral diagnosis

local exfoliated cells (L)・・・local diagnosis

cancer tissue (T)

oral squamous cell cancer : 24 samples

healthy individuals : 2 samples

Cancer suspect patient

Sample preparation

① Obtain informed consent from all study subjects.

② Collect tissue samples (B, L, T).

③ Dissolve samples in 500 µL of lysis buffer, centrifuge.

④ Obtain supernatant of cells.

⑤ Measure protein concentration in these samples.

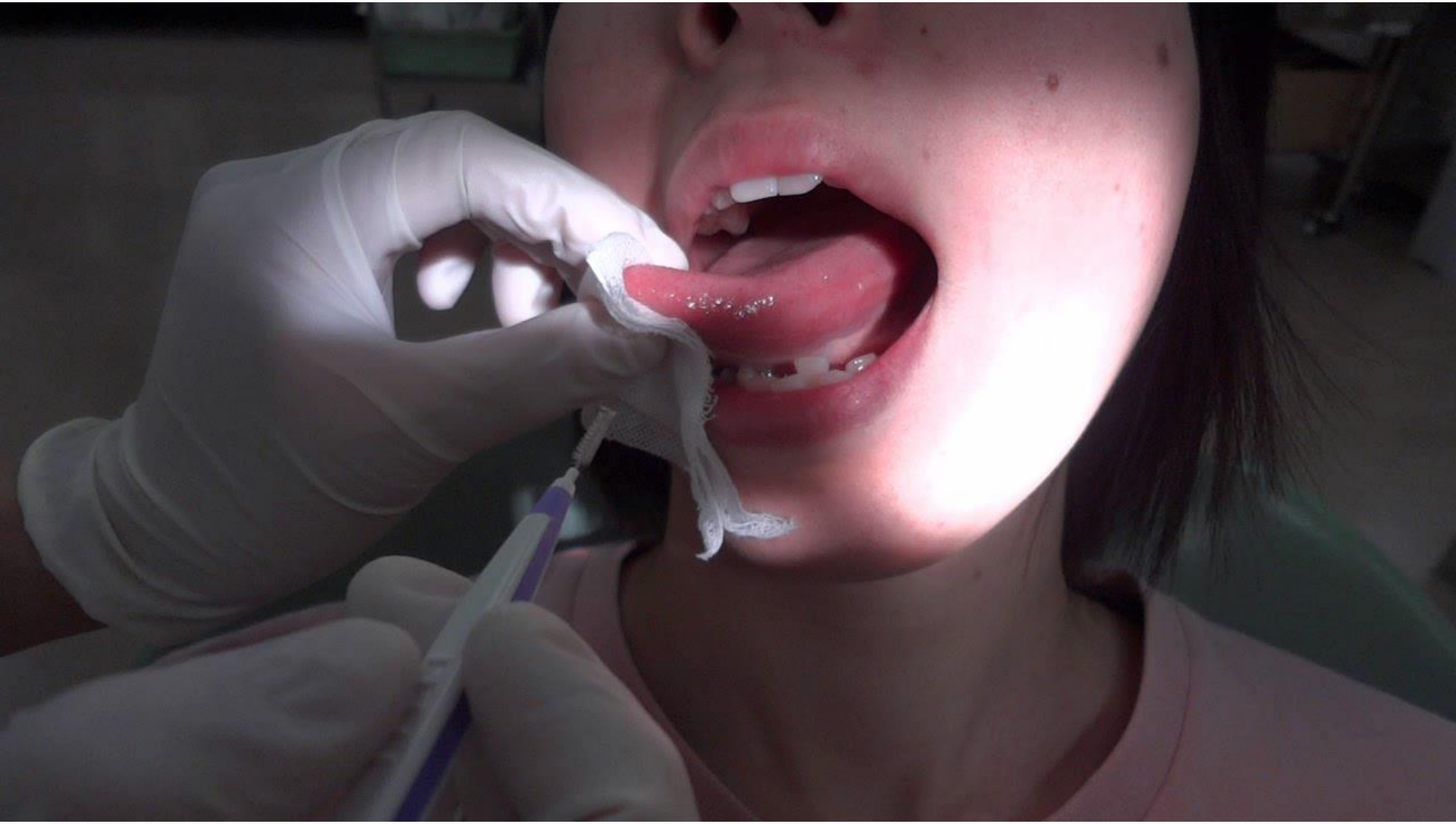
⑥ Adjust concentration（TRAP 2000 ng, Electrochemical method 200 ng).

⑦ Detect telomerase activity by TRAP assay or electrochemical assay.

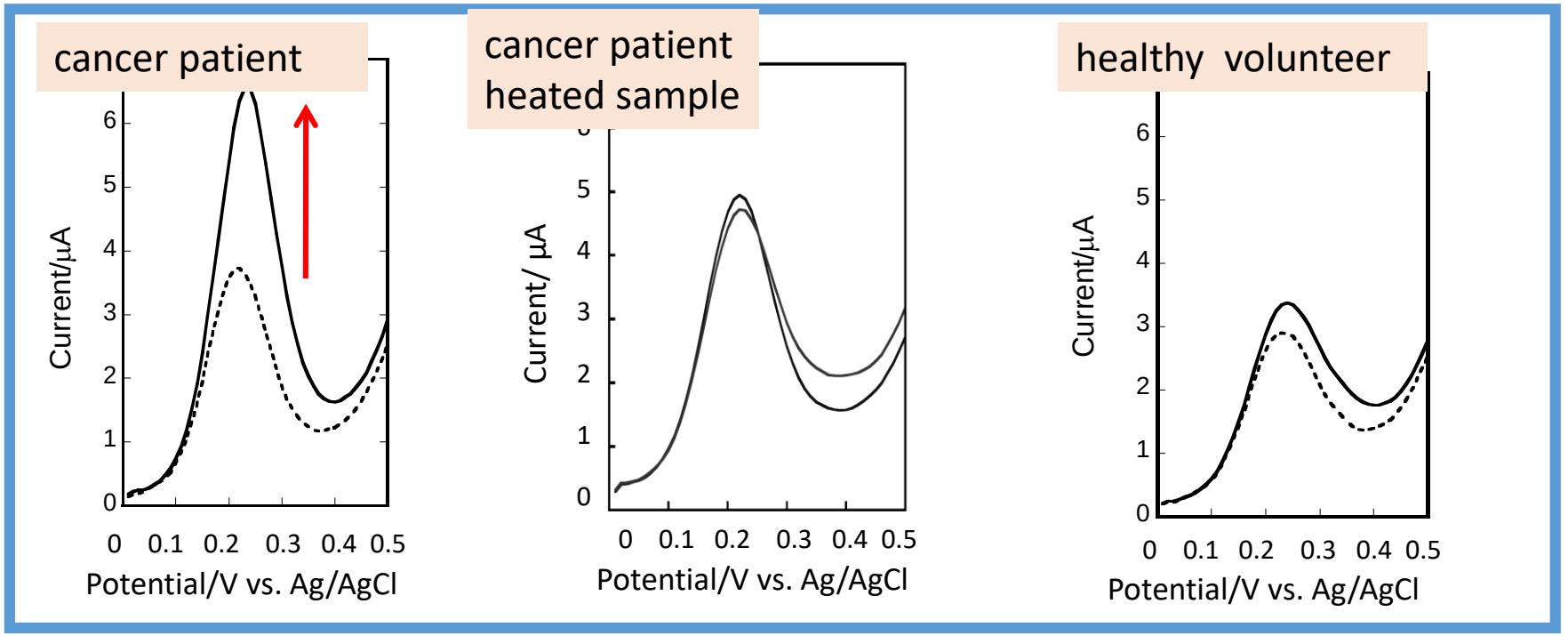
Number of cells : 1×10^7 cells/500 µL

Collecting method of exfoliated buccal cells (B)

Collecting method of local exfoliated cells (L)



SWV results on local exfoliated cells from cancer patient and healthy volunteer



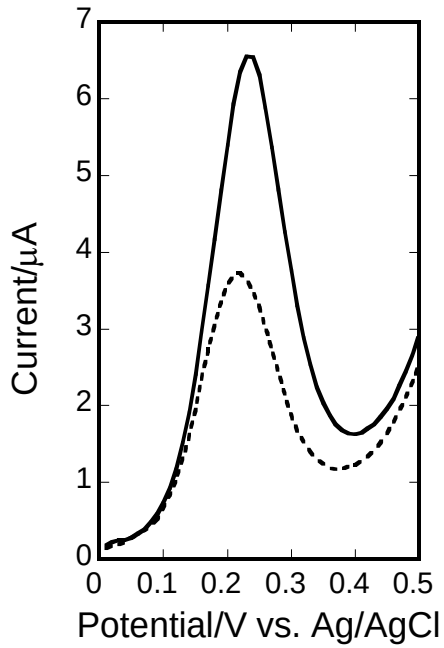
Cancer patient: current increased after treatment

Healthy volunteer: current unchanged before and after treatment

Telomerase extension with clinical samples was successful on the electrode by FND-based electrochemical assay

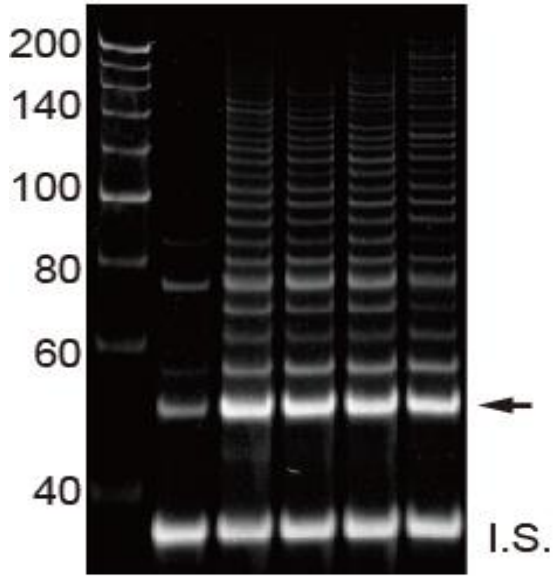
SWV: square wave voltammetry

The calculation of the positive rate in ECTA and TRAP



Classified by Δi

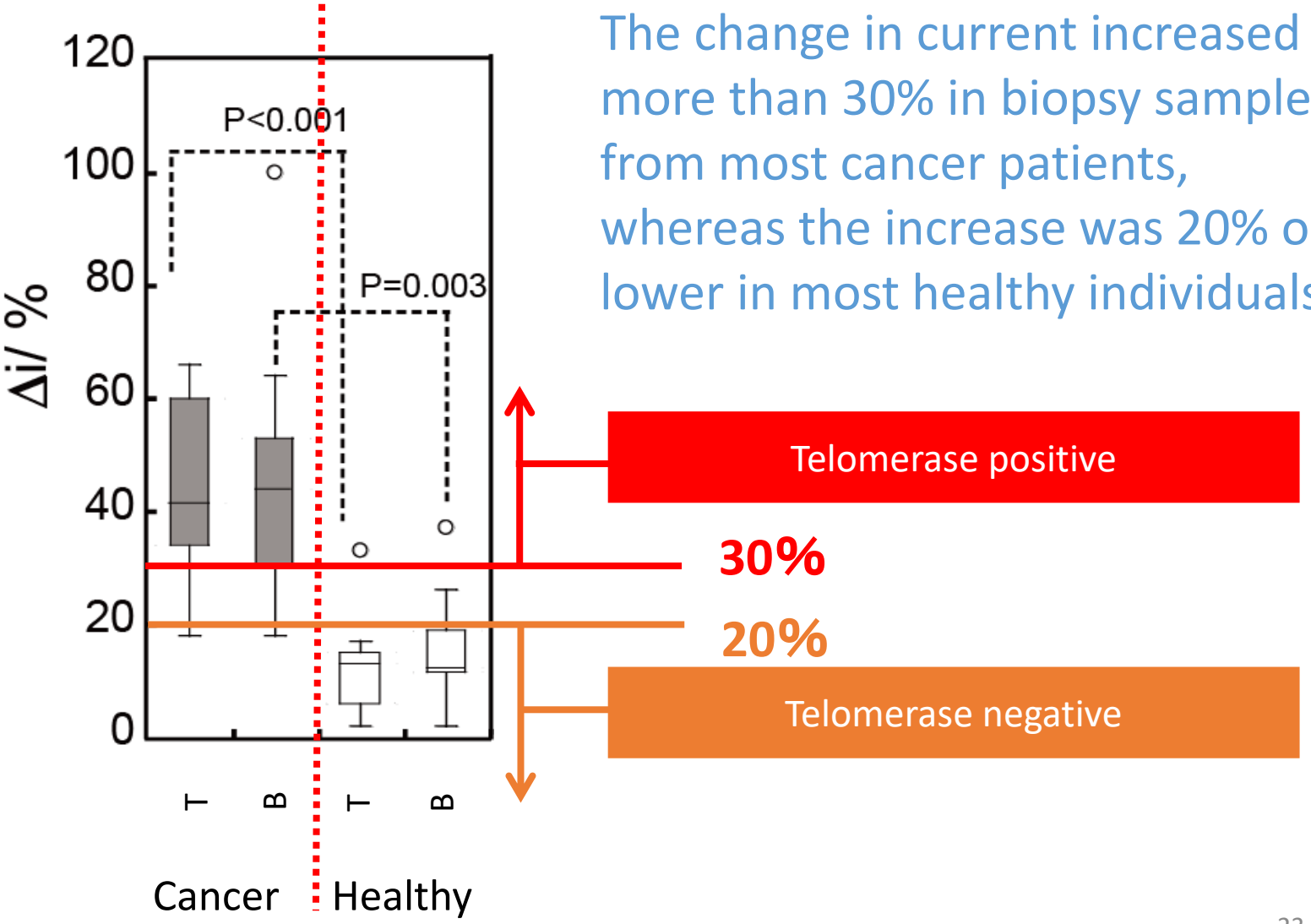
$\Delta i/\% < 20\%$	−
$20\% \leqq \Delta i/\% < 30\%$	±
$\Delta i/\% \geqq 30\%$	+



Classified by number of ladder

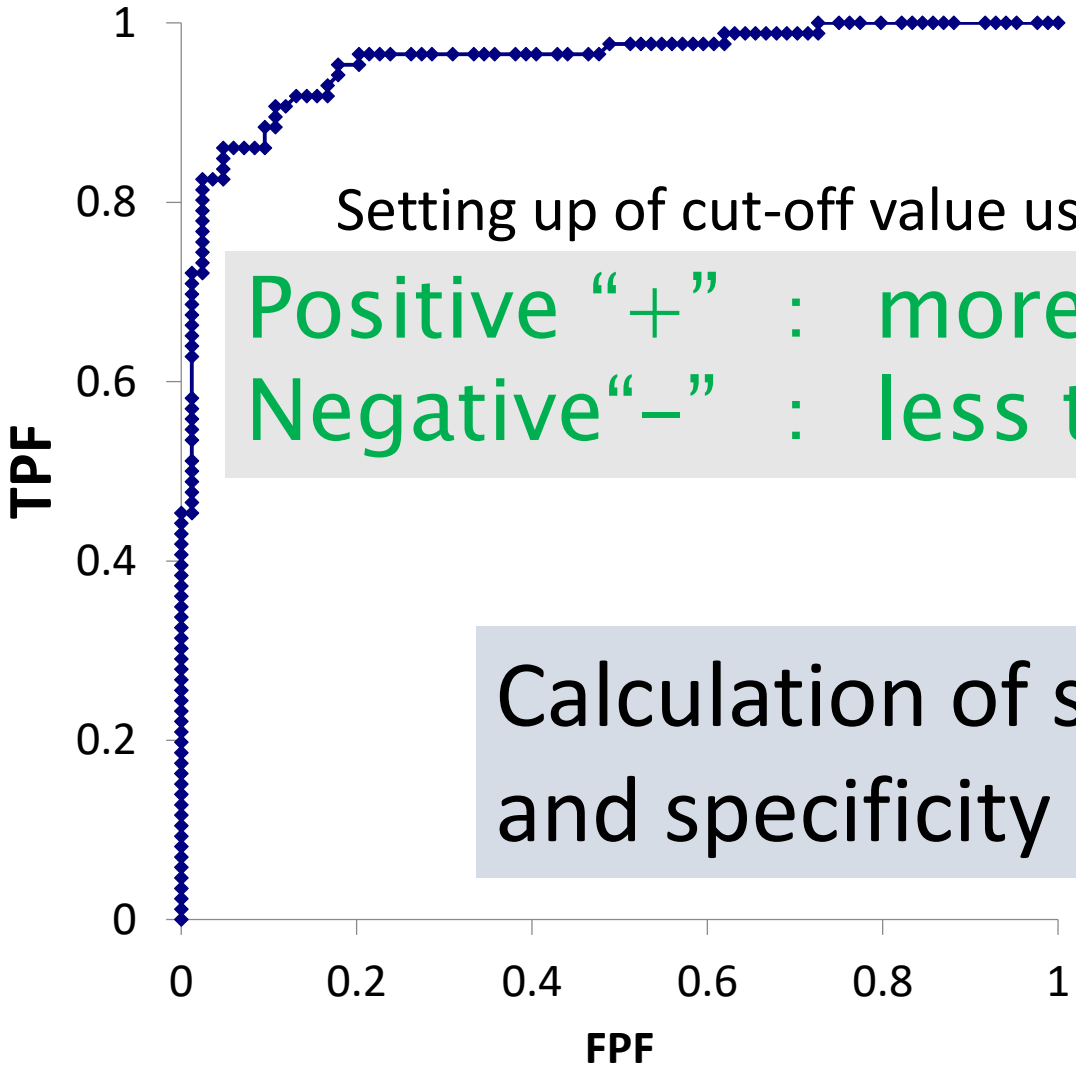
ladder =0	−
0< ladder <5	±
ladder ≧5,	+

ECTA for clinical samples



The change in current increased more than 30% in biopsy samples from most cancer patients, whereas the increase was 20% or lower in most healthy individuals.

Threshold calculation with ROC analysis from ECTA results



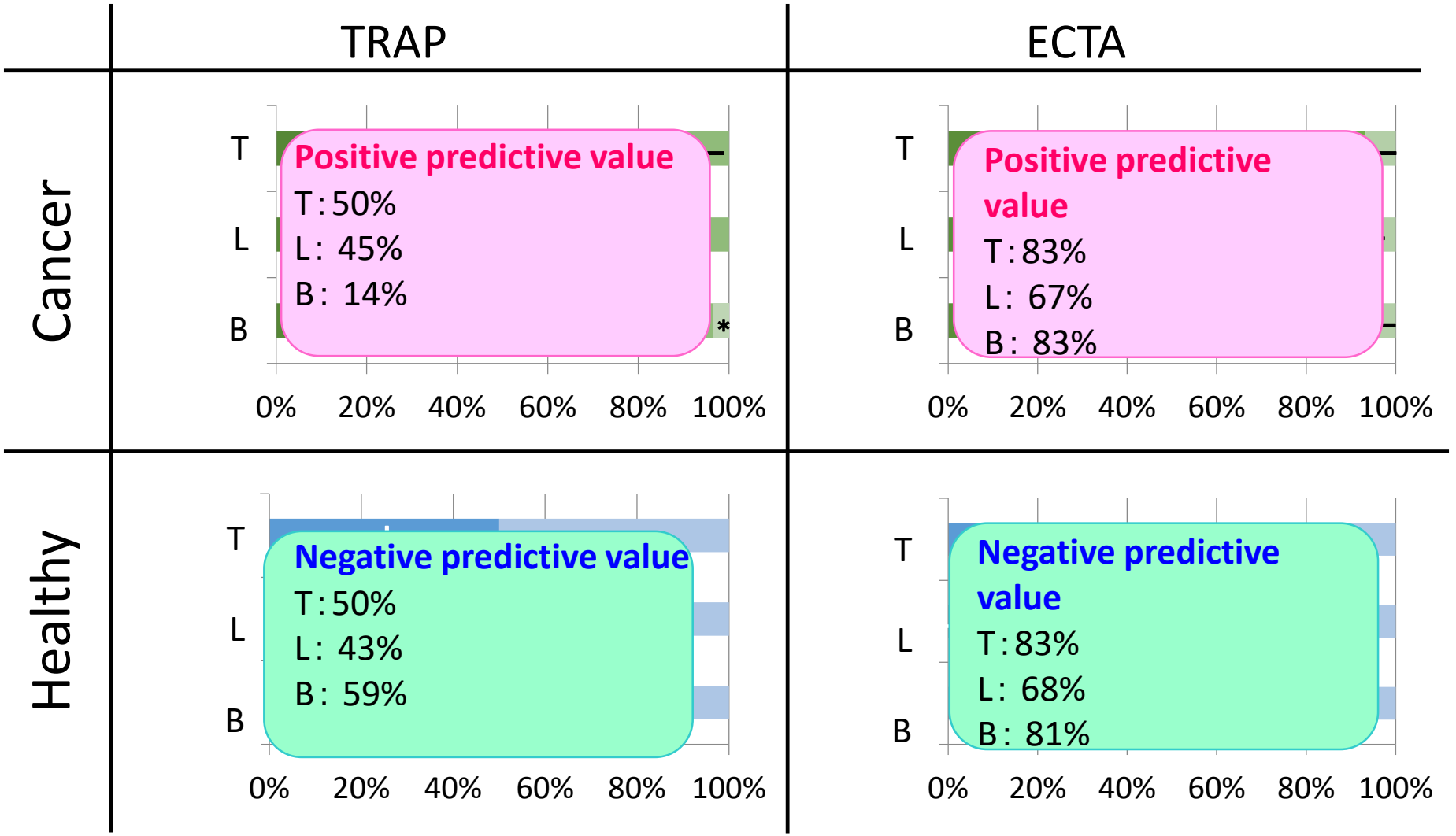
Setting up of cut-off value using ROC analysis

Positive "+" : more than19%

Negative"–" : less than 19%

Calculation of sensitivity and specificity

Calculation of "Positive predictive value using TRAP and ECTA methods



ECTA had lower "±" judgment and estimated telomerase activity with more precision.

Calculation of "Positive predictive value" using ECTA methods

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
T	93%	86%	97%	83%
L	83%	82%	83%	82%
B	93%	86%	97%	83%

ECTA is not only method carrying high sensitivity and specificity for tissue, but also for exfoliated buccal cells (B) and local exfoliated cells (L).

Sensitivity and specificity of cancer diagnosis methods

	Method	Sensitivity / %	Specificity / %	Ref.
Stomach	X-ray	70-80	90	1
Colon	stool analysis	30-93		1
Colon	CEA (carcinoembryonic antigen)	36	87	2
Colon	PAI-1 (plasminogen activator inhibitor1)	94	84	2
Lung	X-ray	63-88	95-99	1
Lung	PET (positron emission tomogram)	70	84	3
Lung	sputum cytology	25-78	99	1
Uterus	Biopsy	95	99	1
Breast	Mammography	84	91	4
Oral	TRAP assay	14	59	5
Oral	Electrochemical Telomerase assay	93	86	5

1) http://ganjoho.jp/med_pro/pre_scr/screening/index.html, (accessed 2017-2-14). 2) N. Sawabu et al., *Pancres.*, **28**, 263 (2004). 3) W. De Wever et al., *Eur. Respir. J.*, **33**, 201 (2009). 4) http://www.bcsc-research.org/statistics/benchmarks/diagnostic/2009/tatableSensSp.html, (accessed 2017-2-14).
5) K. Mori, S. Sato et al., *Clin. Chem.*, **59**, 289 (2013).

Box plot of ECTA result for cancer patient, healthy volunteer, and Leukoplakia, oral lichen planus as benign adenoma