Design of the system

This report is the proposal of a split (binary) probe for DNA analysis based on electrochemistry. The electrochemical signal is modulated by controlled interaction between βCyD and Fc associated with conjugate probe hybridization. The discrimination principle of this system is based on the difference in thermal stability of the shorter half duplex carrying SNP base. Therefore, the length of the half duplex (= one of the conjugate probe, Fc-ODN$_2$ of tandem duplex 2 in Fig. 3 and 4) is a critical parameter to get the signal with high contrast and should be optimized.

Generally, the short DNA probe has high sequence selectivity, but the probability of appearance of the target sequences in a given sample is high. On the other hand, the long DNA probe has high affinity, but is not expected to have sequence selectivity. Split probing is the compromised techniques of the features of both probes. In the present system, the targets are labeled by a pair of the probes. This method allows us to target long sequences without deteriorating the sequence specificity. For the tandem duplex 2, although whole length being targeted is 22-mer (target22N), the recognition of 7-mer sequence by the short probe (Fc-ODN$_2$) solely involves the specificity. Either conjugate probe (Fc-ODN
or βCyD-ODN) could be the one for SNP discrimination that binds/dissociates depending on the kinds of the bases at SNP site.

**General experimental procedures**

All the ODNs used in this study were prepared on a fully automated DNA synthesizer (Expedite 8900, ABI) or purchased from Japan BioServices (Asaka, Japan). The conjugates were synthesized according to our previously reported procedures (see below).3b,6 All the conjugates and targets used in this study were identified by MALDI-TOF MS (Autoflex III, Bruker Daltonics). UV melting experiments and analyses of the curves were performed as previously described.3a,12 Cyclic voltammetries were conducted with printed Au microelectrodes consisting of a pair of interdigitated comb-shaped working electrodes (length 2 mm, width 10 mm, a total of 130 teeth, i.e., 65 from each comb, aligned at 5-mm intervals) and two terminals for counter and reference (Ag/AgCl) electrodes. Square-wave voltammetries were carried out with a standard three-electrode system consisting of a glassy carbon disk (1.6 mmφ), Pt wire, and Ag/AgCl as the working, counter, and reference electrodes, respectively.
Synthesis of CyD-ODN

Monotosylated β-cyclodextrin

β-Cyclodextrin (0.50 g, 0.43 mmol) was dissolved in 4.3 mL of dried pyridine under an atmosphere of argon. To the solution, p-toluene sulfonylchloride (0.16 g, 0.85 mmol) was added in ice bath and then stirred in room temperature. The progress of the reaction was monitored by TLC (1-butanol/ethanol/water = 5/4/3, indicator: p-anisaldehyde) occasionally. The reaction was quenched by an addition of 0.35 mL of water after 3 h. Analysis by TLC indicated the presence of the three spots of β-cyclodextrin ($R_f$ = 0.30), monotosylated- (0.48), and ditosylated-β-cyclodextrin (0.57) in almost the same density. The solution was concentrated to a half in vacuo and poured into 8.5 mL of acetone with vigorous stirring. The resulting white solid was collected and repeatedly recrystallized from water. White solid 72 mg (12.7 %)

$^1$H-NMR (DMSO-$d_6$, 399.65 MHz) $\delta$ 2.43 (s, 3H), 3.10-3.45 (m, 14H), 3.45-3.66 (m, 28H), 4.10-4.60 (m, 6H), 4.76 (s, br, 2H), 4.83 (s, br, 5H), 5.60-5.85 (m, 14H), 7.42 (d, 2H, $J = 8.3$ Hz), 7.74 (d, 2H, 8.3 Hz) ppm
Monothiolated β-cyclodextrin

Monotosylated β-cyclodextrin (0.50 g, 0.39 mmol) and thiourea (0.50 g, 6.6 mmol) were dissolved in 25 mL of 80 % aqueous methanol and refluxed for 72 h at 110 °C. The solution was evaporated in vacuo. The solid was suspended in 7.6 mL of methanol and stirred for 1 h at room temperature. The solid was filtered and dissolved in 17 mL of 10 % aqueous solution of NaOH and stirred for 5 h at 50 °C. After the solution was acidified with 1 M HCl to pH 2, 1.2 mL of trichloroethylene was added to the solution. After stirring overnight, the precipitate was filtered and washed with water. Evaporation of trichloroethylene in vacuo followed by repeated recrystallization from water gave white solid.

White solid 0.27 g (59.4 %)

TLC (silica): one spot (Rf = 0.23, CH₃CO₂Et/n-PrOH/H₂O, 7/7/5)

MALDI-TOF MS calcd for [M+H]⁺: 1150.54, found: 1150.06

SPDP–DNA conjugate

The purified aminopropyl-linked ODN (100 nmol) was dissolved in 100 µL of 0.5 M carbonate-Na buffer (pH 9.3). To this solution, was added SPDP (N-Succinimidyl 3-(2-pyridyldithio)propionate) (1.5 mg, 4.6 µmol) dissolved in 50 µL DMSO. The resulting suspension was stirred at ambient temperature overnight. The solution was diluted to 400 µL with water. The mixture was purified by RP-HPLC under the following conditions: column: Wakosil-II 5C18 RS; room temperature; flow rate: 1.0 mL min⁻¹; eluent A: 0.1 M TEAA (pH 7.0); eluent B: acetonitrile; linear gradient, 5-30 % B in 30 min; detection wavelength: 260 nm. SPDP-DNA conjugate collected were stored at −20 °C after evaporation.

MALDI-TOF MS calcd for [M-H]⁻: 2463.54; found: 2464.43

β-Cyclodextrin–DNA conjugate (CyD-ODN₁)

SPDP–DNA (50 nmol) was dissolved in 100 µL of 10 mM phosphate-Na buffer (pH
7.2). To this solution, was added thiolated β-cyclodextrin (5.8 mg, 5.0 μmol) dissolved in 60 μL DMSO. The resulting suspension was stirred at ambient temperature overnight. The solution was diluted to 400 μL with water. The mixture was purified by RP-HPLC under the following conditions: column: Wakosil-II 5C18 RS; room temperature; flow rate: 1.0 mL min⁻¹; eluent A: 0.1 M TEAA (pH 7.0); eluent B: acetonitrile; linear gradient, 5-30 % B in 30 min; detection wavelength: 260 nm. CyD-ODN₁ collected were stored at –20 °C after evaporation.

MALDI-TOF MS calcd for [M-H]⁻: 3501.87; found: 3501.08
Synthesis of βCyD-ODN conjugate with alkyl chain

βCyD-ODN conjugate with alkyl chain was synthesized according to Scheme S2.

MALDI-TOF MS calcd for [M-H]⁻: 3474.88; found: 3474.89

Scheme S2
Synthesis of Fe-ODN₁

Activated ester of ferrocene (Fc-act)

Ferrocenecarboxylic (0.25 g, 1.1 mmol) acid and N-hydroxysuccinimide (0.15 g, 1.3 mmol) were dissolved in 10 mL of dioxane. Dicyclohexylcarbodiimide (0.25 g, 1.25 mmol) was dissolved in 3 ml dioxane. Both solutions were mixed with stirring and stirred at room temperature for 24 h. The reaction mixture was filtered to remove the precipitate. The filtrate was concentrated to dryness and the solid obtained was chromatographed on a silica gel column.

Yellow solid 0.30 g (85 %)

\[
\text{\textsuperscript{1}H-NMR (CDCl}_3, 399.65 \text{ MHz}) \delta 2.88 \text{ (s, 4H)}, 4.39 \text{ (s, 5H)}, 4.56 \text{ (m, 2H)}, 4.94 \text{ (m, 2H) ppm; IR (KBr)} 1770, 1740, 1220, 1080 \text{ cm}^{-1}; \text{ Found: C 54.95 \%, H 4.11 \%, N 4.52 \%; Calcd for C}_{15}\text{H}_{13}\text{FeNO}_4: C 55.05 \%, H 3.99 \%, N 4.28 \%}
\]

Ferrocene-ODN conjugate (Fe-ODN₁)

The purified aminohexyl-linked ODN (26 nmol) was dissolved in 20 μL 0.5 M NaHCO₃/Na₂CO₃ buffer (pH 9.0). To this was added 6 μL of a DMSO solution of Fc-act (1.3 μmol). After 10 min sonication of this mixture, in which a yellow precipitate appeared, the suspension was stirred at room temperature overnight. Then, the solution was chromatographed on a NAP10 column. The obtained crude material was further purified by RP-HPLC under the following conditions: column: Wakosil-II
Electronic Supplementary Information

5C18 RS; room temperature; flow rate: 1.0 mL min\(^{-1}\); eluent A: 0.1 M TEAA (pH 7.0); eluent B: acetonitrile; linear gradient, 5-30 % B in 30 min; detection wavelength: 260 nm. \textbf{Fe-ODN}\textsubscript{1} collected were stored at –20 °C after evaporation.

MALDI-TOF MS calcd for [M-H]\(^+\) (\textbf{Fe-ODN}\textsubscript{1}): 6420.17; found: 6419.72
Synthesis of Fc-ODN conjugate with short chain (C3)

Fc-ODN conjugate with trimethylene linker chain was synthesized according to Scheme S4.

MALDI-TOF MS calcd for [M-H]−: 6378.60; found: 6378.12

Scheme S4
UV melting inclusion complex formation

Two kinds of βCyD-ODN conjugates were prepared. One is the conjugate with amide linker chain (CyD-ODN<sub>n</sub> in the manuscript). Another one is the conjugate with simple alkyl chain shown in Scheme S2. Thermodynamic parameters for duplex formation of the former conjugate are shown in Table S1. For the latter conjugate, the UV melting curves (around the meltings at lower temperature) and the thermodynamic parameters extracted from them were shown in Figure S1 and Table S2. The stabilization effect of the tandem duplex comes from the formation of the inclusion complex was slightly lower than that of the former conjugate, CyD-ODN<sub>n</sub>. Therefore, the former conjugate was subjected to the subsequent electrochemical studies as shown in the manuscript. Subtle difference in the structure of the linker chain seems to affect the formation of inclusion complex.

Figure S1 UV melting curves of the βCyD-ODN conjugate with alkyl chain from tandem duplexes
left) βCyD-ODN melting from the tandem duplex, βCyD-ODN /target27/ODN20
right) βCyD-ODN melting from the tandem duplex, βCyD-ODN /target27/Fe-ODN<sub>i</sub>
Melting experiments were carried out in 10 mM phosphate buffer solution (pH 7.0) containing 500 mM KCl. The concentrations of DNA components, the conjugates and the targets, were all 1.0 µM. The solutions were heated at a rate of 0.5 °C/min after undergoing annealing and then equilibration for 30 min at 0 °C. black dots: experimental data, red curve: optimized theoretical curve.
Table S1 Melting temperatures and thermodynamic parameters of duplex formation.

<table>
<thead>
<tr>
<th>tandem duplex with target27</th>
<th>$T_{\text{max}}$ / °C</th>
<th>$\Delta H^\circ$ / kcal mol$^{-1}$</th>
<th>$\Delta S^\circ$ / cal K$^{-1}$mol$^{-1}$</th>
<th>$\Delta G^\circ_{298}$ / kcal mol$^{-1}$</th>
<th>$K_{298}$ / mol L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyD-ODN$_1$/Fc-ODN$_1$</td>
<td>37.4</td>
<td>73.5</td>
<td>-60.4</td>
<td>-167</td>
<td>-10.6</td>
</tr>
<tr>
<td>ODN7/Fc-ODN$_1$</td>
<td>19.7</td>
<td>73.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CyD-ODN$_1$/ODN20</td>
<td>18.7</td>
<td>73.9</td>
<td>-42.1</td>
<td>-117</td>
<td>-7.36</td>
</tr>
<tr>
<td>ODN7//ODN20</td>
<td>24.1</td>
<td>73.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The temperatures of biphasic UV inflections. Higher and lower temperatures of two maxima of 1st derivative curves were shown as $T_{\text{max}}$. *b* $\Delta H^\circ$ and $\Delta S^\circ$ for duplex formation of CyD-ODN$_1$ with half duplexes were determined by curve fitting shown in Fig. 2, inset. $\Delta G^\circ$ and $K$ were calculated from them at 298 K.

Table S2 Thermodynamic parameters of duplex formation of βCyD-ODN conjugate with simple alkyl chain

<table>
<thead>
<tr>
<th>neighbor ODN</th>
<th>$\Delta H^\circ$ / kcal mol$^{-1}$</th>
<th>$\Delta S^\circ$ / cal K$^{-1}$mol$^{-1}$</th>
<th>$\Delta G^\circ$ / kcal mol$^{-1}$</th>
<th>$K_{298}$ / M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified</td>
<td>-41.4</td>
<td>-117</td>
<td>-5.59</td>
<td>6.86 $\times$ 10$^4$</td>
</tr>
<tr>
<td>Fe-ODN</td>
<td>-69.2</td>
<td>-201</td>
<td>-9.37</td>
<td>7.45 $\times$ 10$^6$</td>
</tr>
</tbody>
</table>

1 μM DNA, 10 mM Phosphate buffer (pH 7), 1 M NaCl
UV melting_sequence dependence

The UV melting curves of the duplexes of system 2, CyD-ODN$_2$/target22N/Fc-ODN$_2$, were shown in Figure S2. The duplexes with target22G, target22T, and target22A showed biphasic melting because a mispairing destabilized the short duplex with Fc-ODN$_2$. Meanwhile the melting of Fc-ODN$_2$ from the duplex CyD-ODN$_2$/target22C/Fc-ODN$_2$ almost merged with the melting of CyD-ODN$_2$. The measurements of SWV shown in Figure 4 were carried out at 40 °C (313 K). Only the Fc-ODN$_2$ in CyD-ODN$_2$/target22C/Fc-ODN$_2$ was in the duplex state at this temperature.

The inclusion complex formation jacks up the thermal stability of the duplexes as shown in Fig. 2 in the manuscript. Regarding ΔΔG°, it would correspond to the elongation of several bases and could affect the specificity of hybridization. However, the melting study shown in Figure S2 shows that the Fc-ODN$_2$ can distinguish target22C from target22G, target22A, and target22T at relatively wide range of temperature. That is, the effect of the duplex stabilization due to the inclusion complex formation was trivial and did not essentially affect the recognition.

![Figure S2 UV melting curves of the tandem duplexes of system 2, CyD-ODN$_2$/target22N/Fc-ODN$_2$.](image-url)

The melting curves of the duplexes with target22C, target22G, target22T, and target22A were shown as red, blue, green, and black curves, respectively. Melting experiments were carried out in 10 mM phosphate buffer solution (pH 7.0) containing 500 mM KCl. The concentrations of the conjugates and targets were all 1.0 µM. The solutions were heated at a rate of 0.5 °C/min after undergoing annealing and then equilibration for 30 min at 0 °C.
**SWV linker chain length dependence**

We also prepared the two Fc-ODN conjugates with the linker chains of different length, trimethylene (C3) and hexamethylene (C6: Fc-ODN\(_2\)). As the preliminary experiment, the electrochemical property of both conjugates was compared with each other. SWVs are shown in Figure S3. The electric current obtained for the conjugate with C3 linker was almost one forth of C6. Therefore, the conjugate with C6 linker chain was chosen as Fc-ODN\(_n\) in the studies after that. The flexibility of the linker chain seems to significantly affect contact probability of Fc moiety to the electrode.

![Figure S3](image)

**Figure S3** Square-wave voltammograms of the Fc-ODN conjugates with C3 and C6 (Fc-ODN\(_2\)) linker chains. SWVs were performed with a glassy carbon disc as a working electrodes at 25 °C. A 50-µL solutions containing the Fc-ODN conjugates (30 µM), 10 mM phosphate buffer (pH 7.0), and 500 mM KCl were subjected to measurements in a temperature-controlled shield box. amplitude: 25 mV; potential increment: 4 mV; frequency: 15 Hz. Red: C3; blue: C6 (Fc-ODN\(_1\))
**SWV temperature dependence**

Figure S4 shows the square-wave voltammograms of the duplexes CyD-ODN₂/target22N/Fc-ODN₂ obtained at various temperatures. The contrast in current signal decreased with raising temperature. Then, finally, it gave almost the same magnitude as those of the other three duplexes at 55 °C. This coincides with the result of the melting studies shown in Figure S2.*

*The melting temperatures ($T_{\text{max}}$) under both conditions of Figure S2 and S4 would not be the same because the concentrations of ODNs were different for both experiments. The difference in $T_{\text{max}}$, however, should not be so much; the all $T_{\text{max}}$ values under the conditions of Figure S4 (20 µM) might higher than that of Figure S2 (1 µM) by only several degrees in Celsius.

**Figure S4** Square-wave voltammograms of CyD-ODN₂/target22N/Fc-ODN₂. SWVs were performed with a glassy carbon disc as a working electrodes at 45 °C (a), 50 °C (b), and 55 °C (c). A 50-µL solutions containing the DNA components (20 µM), 10 mM phosphate buffer (pH 7.0), and 500 mM KCl were subjected to measurements in a temperature-controlled shield box. amplitude: 25 mV; potential increment: 4 mV; frequency: 15 Hz. Red: target22C; blue: target22G; green: target22T; black: target22A.
SWV at pH 7.5

We have not carried out the comprehensive investigation of pH effect on electrochemical property. Still, SWV measurement for CyD-ODN2/target22N/Fc-ODN2 was done in HEPES buffer (pH 7.5). It was shown in Figure S5. The result was almost the same with that in phosphate buffer solution (pH 7.0).

![Square-wave voltammograms](image)

**Figure S5** Square-wave voltammograms of CyD-ODN2/target22N/Fc-ODN2. SWVs were performed with a glassy carbon disc as a working electrodes at 40 °C. A 50-μL solutions containing the DNA components (20 μM), 10 mM HEPES buffer (pH 7.5), and 500 mM KCl were subjected to measurements in a temperature-controlled shield box. Amplitude: 25 mV; potential increment: 4 mV; frequency: 15 Hz. Red: target22C; blue: target22G; green: target22T; black: target22A.
Hydrodynamic voltammetry \textit{Fe-ODN}_2

The applied potential for the measurement of flow system (Fig. 4(a) in the manuscript) was determined based on the result of hydrodynamic voltammetry. The voltammogram was shown in Figure S6. The current seems to be saturated around 500 mV. Therefore, the ECD potential in the experiments of Fig. 4(a) was determined to be 500 mV vs. Ag/AgCl.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{FigureS6.png}
\caption{Hydrodynamic voltammogram of \textit{Fe-ODN}_2}
\end{figure}

100 pmol of \textit{Fe-ODN}_2 was dissolved in 100 mM phosphate buffer (pH 7.0) containing 0.75 M NaCl and 5 mg/L EDTA. The solutions were injected to HPLC-ECD at various potentials. Flow rate: 1.0 mL/min. Column: Inertsil AX.