S1. Kinetic model
A two-stage binding model is developed to describe the kinetics of the double-stranded (ds) DNA probe. In this model, a free target molecule first binds to the sticky end of the dsDNA probe and displaces the quencher probe subsequently (Figure S1). This binding scheme is modeled as four reversible binding reactions (equations 1-4).

\[
\begin{align*}
M + Q & \leftrightarrow MQ \\
M + T & \leftrightarrow MT \\
MQ + T & \leftrightarrow MQT \\
MT + Q & \leftrightarrow MQT
\end{align*}
\]

Figure S1. A two-step binding model for describing the kinetics of dsDNA probes.

\(M, Q,\) and \(T\) represent the free donor probe, quencher probe, and target. \(MQ, MT,\) and \(MQT\) are molecular complexes of the donor probe bound to the quencher probe, target, and both quencher probe and target simultaneously. In this two-stage binding model, these reactions are modeled by second-order reaction kinetics. Four reversible reactions, six molecular species, and eight kinetic rate constants are involved to capture the dependences of the kinetics on the quencher probe and the sticky end of the dsDNA probe (equation 5-10).

\[
\begin{align*}
\frac{d[M]}{dt} &= -k_{1+}[M][Q] - k_{2-}[M][T] + k_{1-}[MQ] + k_{2+}[MT] \\
\frac{d[Q]}{dt} &= -k_{1+}[M][Q] - k_{4+}[MT][Q] + k_{1-}[MQ] + k_{4-}[MQT] \\
\frac{d[T]}{dt} &= -k_{2+}[M][T] - k_{3+}[MQ][T] + k_{2-}[MT] + k_{3-}[MQT] \\
\frac{d[MQ]}{dt} &= -k_{3+}[MQ][T] - k_{1-}[MQ] + k_{3-}[MQT] + k_{1+}[M][Q] \\
\frac{d[MT]}{dt} &= -k_{4+}[MT][Q] - k_{2-}[MT] + k_{4-}[MQT] + k_{2+}[M][T] \\
\frac{d[MQT]}{dt} &= -k_{3-}[MQT] - k_{4-}[MQT] + k_{3+}[MQ][T] + k_{4+}[MT][Q]
\end{align*}
\]

where \(k\) is the reaction constant. The subscript number represents the reaction in equations 1-4 and “+” and “−” signs denote the forward and reverse reactions.
In this study, the kinetic responses were simulated using MATLAB R2008a (version 7.6) and Gepasi 3. The initial concentrations of the species and the kinetic rate constants were required for simulating the kinetic responses. The initial conditions were determined based on our experimental conditions. In the experiment, 30 nM donor probes and 60 nM quencher probes were hybridized at 90°C and allowed to cool down slowly in the dry bath incubator for three hours. The initial concentrations of \( M, Q, \) and \( MQ \) applied in the numerical simulation were 0, 30, and 30 nM assuming all the donor probes were hybridized to the quencher probes initially. Similarly, the initial concentrations of \( T, MT, \) and \( MQT \) were 312, 0, and 0 nM. The rate constants were estimated based on reported values and further optimized by fitting with experimental data. The ratio of the forward and reverse rate constants (i.e., the equilibrium constant), \( K \), was constrained by the free energy change of the reaction. The equilibrium constant is given by \( K = e^{-\Delta G/RT} \), where \( R \) is the universal gas constant, \( T \) is the absolute temperature, and \( \Delta G \) is the free energy change. The free energy changes of the reactions were estimated based on the probe sequences at 23°C using the Mfold server. The free energy changes for \( M-T \) and \( M-Q \) binding were estimated to be -33 kcal/mol and -19.3 kcal/mol, respectively. The kinetic rate constants optimized for the dsDNA pair of the 21-base donor probe and the 15-base quencher probe is shown in Table 1 (see Figure 6).

### Table S1. Kinetic rate constants for the 21-base donor-15-base quencher pair.

<table>
<thead>
<tr>
<th>( k_{1+} )</th>
<th>( k_1 )</th>
<th>( k_{2+} )</th>
<th>( k_2 )</th>
<th>( k_{3+} )</th>
<th>( k_3 )</th>
<th>( k_{4+} )</th>
<th>( k_4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate (nM(^{-1})s(^{-1}))</td>
<td>4.0×10(^{-3})</td>
<td>2.2×10(^{-17})</td>
<td>7.5×10(^{-3})</td>
<td>1.9×10(^{-25})</td>
<td>1.3×10(^{-4})</td>
<td>1.3×10(^{-10})</td>
<td>3.1×10(^{-3})</td>
</tr>
<tr>
<td>Unit (s(^{-1}))</td>
<td>2.2×10(^{-17})</td>
<td>7.5×10(^{-3})</td>
<td>1.9×10(^{-25})</td>
<td>1.3×10(^{-4})</td>
<td>1.3×10(^{-10})</td>
<td>3.1×10(^{-3})</td>
<td></td>
</tr>
</tbody>
</table>

### S2. Sensitivity analysis

Eight kinetic rate constants were involved in the two-stage binding model. To determine the sensitivity of these parameters in the overall kinetic responses, we performed a sensitivity analysis by varying a rate constant while holding others unchanged. Based on the parameters determined in Table S1, a rate constant was multiplied by a factor of 0.01, 0.1, 1, 10, and 100 in the sensitivity analysis. This changed the rate constant for four orders of magnitude. The overall kinetic responses of \( MT \), which primarily determined the fluorescence response, were studied (Figure S2). Generally, the kinetic responses were not sensitive to the rate constants \( k_{1+}, k_1, k_{2+}, k_{2}, k_{3}, \) and \( k_{4+} \) in the range tested. The kinetic data were overlapped on each other and can not be distinguished in the graphs. For \( k_{3+} \), increasing the binding rate constant showed negligible effect on the kinetic responses and decreasing the value induced significant delay on the kinetics (Figure S2e). Mathematically, the kinetic response was only slow down when the displacement rate was small compared to the target binding rate (i.e., \( k_{3+} << k_4 \)). Physically, this indicated that the reaction kinetics was dominated by the displacement reaction. In such a situation, the kinetic response curve would display a slow growing period initially. This slow growing period was not observed for probes with long sticky ends (>6 bases). Therefore, the time limiting step was likely the displacement reaction for a probe with a long sticky ends. In fact, the kinetic response was highly sensitive to the displacement rate constant \( k_4 \) (Figure S2h). The time scale for the dsDNA assay ranged from seconds to hours. This further indicated the importance of the displacement reaction. Based on this sensitivity analysis, we concluded that the kinetics of dsDNA assays were mainly determined by the binding rate constant \( (k_{3+}) \) and the displacement rate constant \( (k_4) \). These parameters were used for the curve fitting with the experimental data.
Figure S2. Sensitivity analysis of the kinetic rate constants in the two-stage binding model.
S3. Model fitting for estimating rate constants $k_{3^+}$ and $k_{4^-}$.

The rate constants $k_{3^+}$ and $k_{4^-}$, which were found to be most sensitivity to the kinetics responses, were determined based on numerical fitting with the experimental data. These constants were applied to fit all the kinetic data of different quencher lengths and donor lengths using Matlab. To fit the data, the displacement constant, $k_{4^-}$, was assumed to be only determined by the length of the quencher. For instance, an 18-base quencher probe would have the same displacement constant when pairing with 21-base or 24-base donor probes, as the sequence involved in the displacement reaction was the same. Similarly, the binding constant, $k_{3^+}$, was assumed to depend on the length of the sticky end, i.e., the same length of the sticky end has the same binding constant $k_{3^+}$. Strictly speaking, the binding constant depends on the sticky end sequence, instead of the length. This simplifies the fitting procedure and will provide a general guideline in the design of the dsDNA assay. This leads a set of seven binding constants and four displacement constants in our experiments. Tables S2 and S3 show the kinetic rate constants determined by the fitting procedure. Figure S3 shows the comparison of the kinetic responses determined by experiments and the two-stage binding model. The two-stage binding is able to describe the general trend observed in our experiment. As determined in the sensitivity analysis, the kinetic response is only sensitive to the binding constant, $k_{3^+}$, when it is small compared to the displacement rate, $k_{4^-}$. The value $k_{3^+}$ is likely to be more accurate for probes with short sticky ends.

Table S2. The binding constant $k_{3^+}$ for different sticky end lengths

<table>
<thead>
<tr>
<th>Sticky end, bases</th>
<th>3</th>
<th>6</th>
<th>8</th>
<th>9</th>
<th>11</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{3^+}$, nM⁻¹s⁻¹</td>
<td>2.0×10⁻⁷</td>
<td>1.3×10⁻⁴</td>
<td>2.0×10⁻⁴</td>
<td>2.4×10⁻⁴</td>
<td>2.7×10⁻⁴</td>
<td>2.8×10⁻⁴</td>
<td>3.0×10⁻⁴</td>
</tr>
</tbody>
</table>

Table S3. The displacement constant $k_{4^-}$ for different quencher lengths

<table>
<thead>
<tr>
<th>Quencher probe, bases</th>
<th>10</th>
<th>12</th>
<th>15</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{4^-}$, s⁻¹</td>
<td>3.1×10⁻³</td>
<td>2.3×10⁻³</td>
<td>1.3×10⁻³</td>
<td>2.3×10⁻⁴</td>
</tr>
</tbody>
</table>
Figure S3. Fitting of experimental data with the two-stage binding model.
S4. Titration curve

Compared to the previous equilibrium analysis, the two-stage binding model introduced two additional reversible reactions to incorporate the intermediate binding step. This could change the steady behavior of the reactions. We studied the equilibrium response of the model to investigate its accuracy in predicting the steady state behavior of the assay. The 24-base donor probe was pre-hybridized with the 10-base quencher probe to determine the titration curve. A serial dilution experiment was performed to prepare the target at different concentrations. The targets were then mixed with the pre-hybridized probe in 384-well plates. Figure S4 shows the comparison of the titration curves determined by the model and experimental data. The result is in good agreement, which demonstrates the applicability of the model for predicting the steady stage response of the assay.

Figure S4. Comparison of the titration curves determined by (a) the two-stage binding model and (b) experiment investigation.
**S5. Model fitting for ionic strengths**

The dependence of the reaction rate constants on the ionic strength was determined by fitting the experimental data with the two-stage binding model. Since the reaction kinetics was dominated by the displacement reactions, the kinetics was only sensitive to the displacement rate constant, $k_{4-}$, in the fitting procedure.

![Figure S5](image1.png)

Figure S5. Fitting of kinetic responses at different NaCl concentrations. Kinetics determined by (a) the two-stage binding model and (b) experiment.

![Figure S6](image2.png)

Figure S6. Dependences of the disassociation rate constant, $k_{4-}$, as a function of the salt concentration.