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Noncooled Capillary Inlet: A Source of Systematic Errors in Capillary-Electrophoresis-Based Affinity Analyses

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Capillary electrophoresis (CE) serves as a platform for a large family of temperature-sensitive affinity methods. To control the electrolyte temperature, the heat generated during electrophoresis is removed by actively cooling the capillary. Short parts of the capillary, particularly at its inlet, are not actively cooled, however, and the electrolyte in this part is likely to be at an elevated temperature. Owing to their relatively short lengths, the noncooled parts have never been considered as a potential source of artifacts. Here we report for the first time that electrophoresis of the sample through the short noncooled capillary inlet can lead to large systematic errors in quantitative CE-based affinity analyses. Our findings suggest that the noncooled capillary inlet region, in spite of being short, is a source of significant artifacts that must be taken into consideration by developers and users of CE-based affinity methods. We propose a simple solution for this problem: moving the sample through the noncooled inlet into the cooled region by pressure or by a low-strength electric field to save it from exposure to the elevated temperature.

Capillary electrophoresis (CE) has found multiple uses in affinity analyses. The major applications of CE-based affinity methods include (i) quantitative analysis of the target concentration through its binding to a labeled affinity probe,[1−5] (ii) determination of binding parameters of noncovalent affinity complexes,[6−13] and (iii) selection of affinity ligands, such as DNA aptamers.[14−18] Affinity analyses are highly sensitive to temperature as a temperature change of only a few degrees can lead to significant changes in kinetic and thermodynamic parameters of affinity interactions.[19,20] It is thus essential to remove heat from the electrolyte and keep its temperature as well controlled as possible.

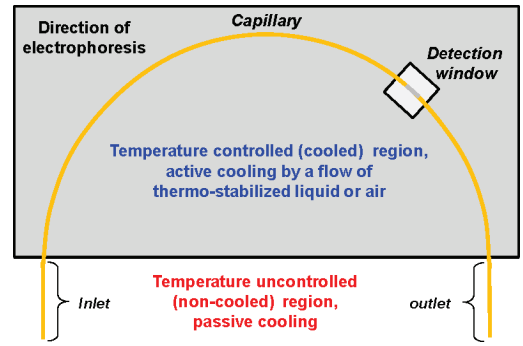

Figure 1. Schematic representation of a capillary cartridge assembly of a standard commercial CE instrument. The major portion of a capillary is actively cooled by a thermostated heat exchanger which can be a flow of air or liquid, or the capillary may be in contact with a metal surface. The inlet and outlet regions of the capillary, as well as the detection window, are inserted into the running buffer vials, are hanging in the air, or are in contact with the rubber or plastic instrument interface and are not subjected to any form of temperature control.

To remove the heat and control the electrolyte temperature, CE instruments are typically equipped with capillary cooling systems. A capillary, for most of its length, is placed in contact with a thermostabilized heat exchanger (Figure 1). Short parts of the capillary at its inlet, outlet, and detection window are not, however, in contact with the heat exchanger. They are not actively cooled and can, thus, have higher temperatures. Temperature measurements in CE were performed using different approaches

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by a number of groups,[21−28] and the difference in temperatures of the cooled and noncooled capillary regions was suggested by Hruska et al.[29] However, the temperature in the noncooled capillary regions has never been determined, and its influence on CE-based affinity analyses has always been ignored.

This work was motivated by a hypothesis that the noncooled inlet of the capillary, even being short, can potentially affect the accuracy of CE-based affinity analyses. Indeed, if the temperature of the noncooled inlet is elevated, affinity complexes can considerably dissociate even during the short time of their passing through the noncooled part. Such dissociation can, in turn, affect quantification in affinity analyses. To demonstrate this, we used two "test" affinity methods. The first method was nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), which facilitates the determination of equilibrium and rate constants of complex dissociation: K_d and k_{off}, respectively. The second method was quantitative DNA hybridization assay, which determines the concentration of the target DNA using a labeled DNA probe. We found that the elevated temperature in the inlet region resulted in the overestimation of K_d and k_{off} values in one example of NECEEM-based measurements by factors of 40 and 3, respectively. In the CE-based hybridization assay the temperature-elevated inlet region led to underestimation of the target concentration by a factor of 3. This finding indirectly suggests that the temperature in the noncooled inlet part is significantly higher than in the cooled part. It also suggests that other CE analyses with temperature-sensitive samples can be affected in a similar way. Fortunately, the problem has a very simple solution: the systematic errors can be completely eliminated by simply moving the sample through the noncooled inlet region by either pressure or a low-strength electric field. Finally, our finding emphasizes the need for the development of approaches for temperature determination in cooled and noncooled parts of the capillary which are currently not available.

RESULTS AND DISCUSSION

In general, there are three noncooled parts of the capillary in CE with capillary thermostabilization: the inlet, the outlet, and the detection window (Figure 1). The analytical separation is complete for every analyte by the time it reaches the detection window and capillary outlet. Therefore, we do not expect that the elevated temperature in the noncooled detection window and capillary outlet can significantly affect CE-based affinity analyses (preparative separation may be an exception). Therefore, the focus of this work was the noncooled region of the capillary inlet.

To study the effect of sample electromigration through the noncooled inlet on the CE analysis, one needs to have a means of avoiding such an effect. Fortunately, the solution for this problem is very simple. To avoid sample exposure to the potentially elevated temperature in the noncooled inlet part, one simply needs to move the sample through the noncooled part to the cooled region by pressure under a zero electric field. Thus, we compared the results of two types of experiments. In the first type of experiment, an electric field was applied immediately after the sample was injected into the capillary by pressure so that the sample moved through the noncooled region under a potentially significantly elevated temperature. In the second type of experiment, the sample was propagated through the noncooled part by pressure under an ambient (room) temperature; the electric field was applied only when the sample safely reached the actively cooled region.

Using the above approach, we studied the potential effect of the noncooled inlet region on CE-based quantitative affinity analyses. The first test method used here was NECEEM.[6,7] In NECEEM, a short plug of the equilibrium mixture of two interacting molecules is injected into the capillary and its components (unbound molecules and the complex) are separated by electrophoresis. The separation promotes complex dissociation. The resulting NECEEM electropherogram has a "memory" of equilibrium and also records the dissociation process. Accordingly, NECEEM allows the calculation of rate (k_{off}) and equilibrium (K_d) constants of complex dissociation from a single electropherogram.[6] The rate constant of complex formation (k_{on}) can then be calculated as k_{off}/K_d. See the Supporting Information for details on calculating K_d and k_{off} in NECEEM.

The pair of interacting molecules subjected to NECEEM was single-stranded DNA binding protein (SSB) and a 15-nt-long ssDNA of a scrambled sequence. DNA was fluorescently labeled for detection. The results of NECEEM experiments with and without the sample's exposure to the potential elevated temperature in the noncooled inlet are shown in Figure 2. Simple visual comparison of the two electropherograms reveals a striking difference between them. Electrophoresis at 600 V/cm through the noncooled region (upper electropherogram) leads to the apparent decrease in the intact complex and increase in unbound (free) DNA. This unambiguously indicates that Joule heating raises the temperature in the noncooled region to the level that considerably speeds the SSB−DNA complex dissociation. When the complex rapidly dissociates in the short noncooled inlet, it produces an exponential dissociation segment in the electropherogram. This segment is so steep and short that it cannot be resolved from the peak of unbound DNA and contributes to this peak's area. Therefore, the "free DNA" peak in the top electropherogram appears very large. Accordingly, the peak of the intact complex in the top electropherogram is very small. Importantly, the overestimation of the area of the free DNA peak leads to the overestimation of K_d values. For our SSB−DNA experiment, the exposure to the elevated temperature in the noncooled inlet resulted in a 40-fold overestimation of the K_d value. The accuracy of k_{off} calculation was not affected much since it did not rely on the peak of free DNA. The value of k_{off} is calculated using the areas of the peaks of the intact and dissociated complex. These areas are mainly formed in the cooled part of the capillary. The value of k_{off} was overestimated by a factor of 3.

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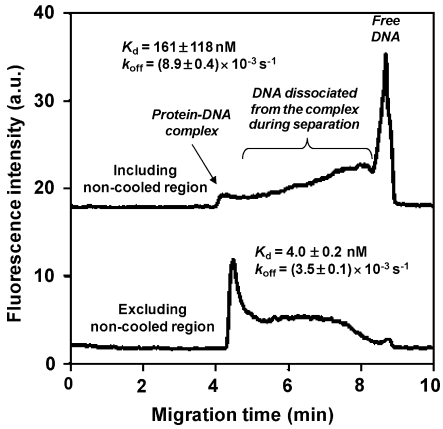

Figure 2. Effect of the noncooled capillary inlet on NECEEM-based determination of rate (k_{off}) and equilibrium (K_d) constants of the protein−DNA interaction. The equilibrium mixture contained 500 nM SSB and 250 nM 15-nt-long fluorescently labeled ssDNA of a scrambled sequence. The run buffer was 50 mM Tris−acetate at pH 8.3. Separations were performed at 600 V/cm with a 20 °C temperature of the capillary coolant. The bottom electropherogram corresponds to NECEEM analysis excluding the noncooled inlet region of the capillary. The top electropherogram corresponds to the NECEEM analysis including the noncooled capillary inlet. The exclusion of the noncooled region shortens the effective capillary length, which alters the migration time of the components. To simplify the comparison of the electropherograms, the time scale for the experiment with an excluded noncooled region was multiplied by a factor of 1.14, which is the ratio between the effective capillary lengths.

To confirm that the observed effect was not unique to the SSB−DNA interactions, we used NECEEM to measure K_d and k_{off} for the interaction between MutS protein and its DNA aptamer. The results were similar: the elevated temperature in the noncooled inlet also led to overestimated K_d and k_{off}. To demonstrate that the degree of the effect of sample heating depends on the CE run buffer ionic composition, we used a run buffer with different electric conductivities (with and without 25 mM KCl) (Figure 3). The effect of the noncooled region was more pronounced when the run buffer contained KCl. Such a result was expected since the KCl-containing buffer has a greater conductivity and, thus, generates more heat, leading to a greater temperature increase in the noncooled inlet region. It is important to mention that NECEEM and its earlier versions are also used for quantitative analyses of the target concentration through its binding with the labeled affinity probe.[1,6] The calculations in such analyses include the peak area of the equilibrium fraction of the unbound probe. Accordingly, the elevated temperature in the noncooled capillary inlet should cause systematic errors in calculated concentrations similar to the errors in K_d in our above examples.

The second test method used in this work was CE-based DNA hybridization assay. In such an assay, a hybridization mixture is prepared by mixing the target DNA of unknown concentration with a labeled DNA probe at a higher known concentration. The values of K_d for DNA hybridization at temperatures considerably below the melting temperature are very low. Accordingly, when the probe hybridizes the target, no unbound target is left while the excess of the probe is present in the hybridization mixture. A short plug of the hybridization mixture is injected into the capillary, and the unbound ssDNA probe is separated from the dsDNA probe−target hybrid. The unknown concentration of the target DNA is found from the areas of the peaks of the unbound probe and the hybrid (see the Supporting Information for details of the calculations).[30]

The DNA target analyzed by a CE-based hybridization analysis was 15-nt-long ssDNA with a relatively high GC content of 80% and, accordingly, a relatively high melting temperature for the hybrid of about 40 °C as estimated by the IDT OligoAnalyzer 3.1 software. The DNA probe with a sequence complementary to that of the target was fluorescently labeled for detection. The results of DNA hybridization experiments with and without exposing the hybridization mixture to the elevated temperature in the noncooled inlet are shown in Figure 4. The electropherograms reveal the obvious difference: electrophoresis through the noncooled region

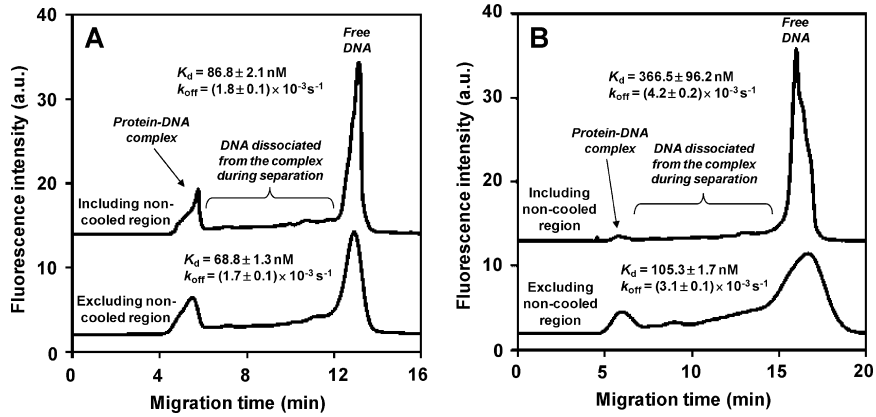

Figure 3. Effect of the noncooled capillary inlet on NECEEM-based determination of rate (k_{off}) and equilibrium (K_d) constants of the MutS protein interaction with its fluorescently labeled DNA aptamer in run buffers with low (A) and high (B) electric conductivity. The high-conductivity buffer was similar to the low-conductivity buffer (50 mM Tris−acetate, 2.5 mM $MgCl_2$, pH 8.3) but contained 25 mM KCl. The equilibrium mixture contained 100 nM MutS and 50 nM aptamer. Separations were performed at 600 V/cm with a 20 °C temperature of the capillary coolant. The bottom electropherograms correspond to NECEEM excluding the noncooled inlet region of the capillary. The top electropherograms correspond to NECEEM including the noncooled inlet region. Since the effective length of the capillary used for electrophoresis was shorter when the sample was propagated by pressure in the first 5 cm, the exclusion of the noncooled region shortens the effective capillary length, which alters the migration time of the components. To simplify the comparison of the electropherograms, the time scale for the experiment with the excluded noncooled region was multiplied by a factor of 1.33, which is the ratio between the effective capillary lengths.

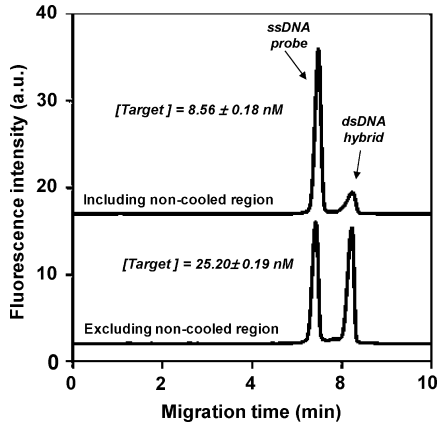

Figure 4. Effect of the noncooled capillary inlet on the DNA hybridization assay. The hybridization mixture contained 25 nM 15-nt-long ssDNA target and 50 nM complementary fluorescently labeled DNA probe. The CE run buffer was 25 mM sodium tetraborate buffer at pH 9.3. Separations were performed at 600 V/cm with a 20 °C temperature of the capillary coolant. The bottom electropherogram corresponds to CE separation excluding the noncooled capillary inlet. The top electropherogram corresponds to CE separation including the noncooled capillary region. The exclusion of the noncooled region shortens the effective capillary length, which alters the migration time of the components. To simplify the comparison of the electropherograms, the time scale for the experiment with the excluded noncooled region was multiplied by a factor of 1.14, which is the ratio between the effective capillary lengths.

(upper electropherogram) leads to the apparent decrease in the amount of the hybrid and increase in the amount of unbound (free) probe. This result confirms that Joule heating raises the temperature in the noncooled region to a level that considerably speeds melting of the DNA hybrid. On the other hand, the overestimated peak area of the unbound probe and underestimated peak area of the hybrid lead to systematic errors in the value of the target concentration calculated from the areas. In the example shown in Figure 4, the electrophoresis at 600 V/cm through the noncooled inlet resulted in underestimation of the target concentration by a factor of 3. This effect is expected to be even more significant in hybridization assays for RNA targets using DNA probes, since DNA−RNA hybrids are less temperature-stable than dsDNA of the same sequence.

CONCLUDING REMARKS

Our results suggest that Joule heating can significantly increase the electrolyte temperature in the short noncooled capillary inlet while not significantly affecting the temperature in the cooled region. The heated electrolyte, in turn, can dissociate affinity complexes and lead to large systematic errors in CE-based quantitative affinity analyses. In this proof-of-principle work, we used two CE-based affinity methods, NECEEM and DNA hybridization assay, to demonstrate the influence of Joule heating in the noncooled capillary inlet. Other CE-based affinity methods will also be affected, although the extent of the influence of the noncooled region will depend on the method and the sample. Sample heating in the noncooled inlet can also affect other CE methods, specifically those with temperature-sensitive analytes, such as proteins.

Retrospectively, CE-based affinity methods have been in use for nearly two decades. In some works, systematic errors due to noncooled regions could have been relatively small, since smaller diameter capillaries and lower strength electric fields were used; in other works, the errors could be significant. Remeasuring all previous results is impractical, but it is likely possible to develop an approach for assessing potential errors on the basis of the conditions used (type of run buffer, electric field strength, capillary diameter, etc.) at least for commercial CE instruments with standard cooling systems. It is more important, however, to ensure that such systematic errors are avoided in the future. This emphasizes the need for further study of the phenomenon and for the development of experimental methods capable of measuring temperature in the noncooled capillary inlet.

MATERIALS AND METHODS

Chemicals and Materials. Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). The HPLC-purified, fluorescently labeled MutS aptamer 5′-fluorescein-CTT CTG CCC GCC TCC TTC CTC GGG GTT AGA ACG TCG TGT AGG ACT CCT ATC GGT TTA TGG AGA CGA GAT AGG CGG ACA CT, fluorescently labeled 15-nt-long scrambled ssDNA probe 5′-Alexa488-GCG GAG CGT GGC AGG, and 15-nt-long ssDNA target 5′-CCT GCC ACG CTC CGC-3′ complementary to the probe were purchased from IDT DNA Technology Inc. (Coralville, IA) and dissolved in a TE buffer (10 mM Tris−HCl, 0.1 mM EDTA, pH 7.5). All other reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless stated otherwise. All solutions were made using deionized water filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada).

Instrumentation. All experiments were conducted with an MDQ-PACE instrument (Beckman-Coulter) equipped with a fluorescence detector; a 488-nm solid-phase laser was used to excite fluorescence. The CE instrument employed had a capillary temperature control system: the outer walls of the capillary were in contact with a liquid heat exchanger, maintained at a fixed temperature. Bare fused-silica capillaries were used in all experiments to induce an electroosmotic flow. The inner and outer diameters and the length of the capillary were 75 μm, 360 μm, and 50 cm, respectively. The length from the injection end (inlet) to the detection window was 40 cm. The length of the noncooled inlet region was 4.5 cm. The temperature of the heat exchanger was controlled by the instrument with a precision of ±0.1 °C (as per the manufacturer's specifications).

Electrophoresis Conditions. The CE run and sample buffers used were identical. For NECEEM experiments of SSB binding DNA the buffer was 50 mM Tris−acetate at pH 8.3. For NECEEM experiments of MutS binding its DNA aptamer the buffer was 50 mM Tris−acetate at pH 8.3 supplemented with 2.5 mM $MgCl_2$ and either containing 25 mM KCl or free of KCl. For DNA hybridization experiments the buffer was 25 mM sodium tetraborate at pH 9.2. The sample was introduced by a 0.5 psi (3.4 kPa) pressure pulse for 5 s. To exclude the effect of the noncooled inlet region on the NECEEM experiments, the injection step was followed by a second pressure pulse of 0.3 psi (2.07 kPa) for 1 min for SSB−DNA and 3 min for MutS−aptamer pairs. The second pressure pulse moved the sample past the first 5−10 cm of capillary length to its cooled region. The longer duration of the pressure pulse for the

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MutS−aptamer pair was required to compensate for the observed decrease in the velocity of pressure-driven sample propagation in the presence of $MgCl_2$ and KCl in the buffer. To exclude the effect of the noncooled inlet region on the DNA hybridization experiments, the hybridization mixture was initially moved by the electroosmotic flow at a low electric field strength of 100 V/cm for 6 min to the actively cooled capillary region. The second pressure pulse was not used for the hybridization analysis since it decreases the resolution of the ssDNA probe from the dsDNA hybrid. Prior to each run, the capillary was rinsed with 100 mM NaOH, 100 mM HCl, and deionized water for 2 min each, followed by a 2 min rinse with the run buffer using a pressure of 20 psi (68 kPa). The order of experiments with including and excluding noncooled regions was shuffled to avoid any potential reagent-stability artifacts.

NECEEM of SSB−ssDNA Interactions. The equilibrium mixture of 250 nM fluorescently labeled 15-nt scrambled ssDNA and 500 nM SSB was prepared in 50 mM Tris−acetate buffer at pH 8.3 and incubated overnight at 4 °C to reach equilibrium. An aliquot of the equilibrium mixture was then incubated for 15 min at room temperature and used in an NECEEM experiment. A fresh refrigerated aliquot was taken for each subsequent experiment.

NECEEM of MutS−Aptamer Interaction. The MutS aptamer was first prepared at a concentration of 1 μM in 50 mM Tris−acetate buffer at pH 8.3, supplemented with 2.5 mM $MgCl_2$ and either containing or not containing 25 mM KCl. The aptamer was "annealed" in a thermocycler by heating it to 80 °C for 3 min and then cooling it to 20 °C at a rate of 7.5 °C/min. The annealed aptamer and MutS were then mixed in the same buffer to reach their concentration of 50 and 100 nM, respectively. The mixture was incubated at 4 °C for 7 h to allow for equilibrium to be established. An aliquot of the equilibrium mixture was then incubated for 15 min at room temperature and used in NECEEM experiments. A fresh refrigerated aliquot was taken for each experiment.

DNA Hybridization Assay. The hybridization mixture of 50 nM fluorescently labeled 15-nt scrambled ssDNA probe and 25 nM 15-nt complementary ssDNA target was prepared in 25 mM sodium tetraborate buffer at pH 9.2. The mixture was then annealed in a thermocycler by heating it to 95 °C for 2 min, then decreasing the temperature to 35 °C at a rate of 7.5 °C/min, holding it at 35 °C for 1.5 min, and finally holding it at 20 °C before use.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Non-Cooled Capillary Inlet – A Source of Systematic Errors in Capillary Electrophoresis-Based Affinity Analyses

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1. Determination of k_{off} and K_d by NECEEM

The equilibrium and the rate constants of complex dissociation, K_d and k_{off}, respectively, were calculated using following equations:

$$K_d = \frac{[\text{Protein}]_0\left\{1 + A_1/(A_2 + A_3)\right\} - [\text{DNA}]_0}{1 + (A_2 + A_3)/A_1} \qquad \textbf{(S1)}$$

$$k_{off} = \frac{\ln\left\{(A_2 + A_3)/A_2\right\}}{t_C}$$

Here A_1, A_2, and A_3 are the areas which correspond to: (i) free DNA which was unbound in the equilibrium mixture, (ii) protein-bound DNA, and (iii) DNA that dissociated from the protein-DNA complex during electrophoresis. The areas are illustrated in the example of a NECEEM electropherogram presented in **Fig. S1**. The other parameters are: t_c is the time that complex spends dissociating during the separation and $[\text{Protein}]_0$ and $[\text{DNA}]_0$ are the total concentrations of the protein and DNA in the equilibrium mixture. The detailed derivations of the above equations are described elsewhere (references 6 and 7 in main text). The bimolecular rate constant of complex formation (k_{on}) can be then calculated using the determined values of k_{off} and K_d:

$$k_{on} = k_{off} / K_d \qquad \textbf{(S2)}$$

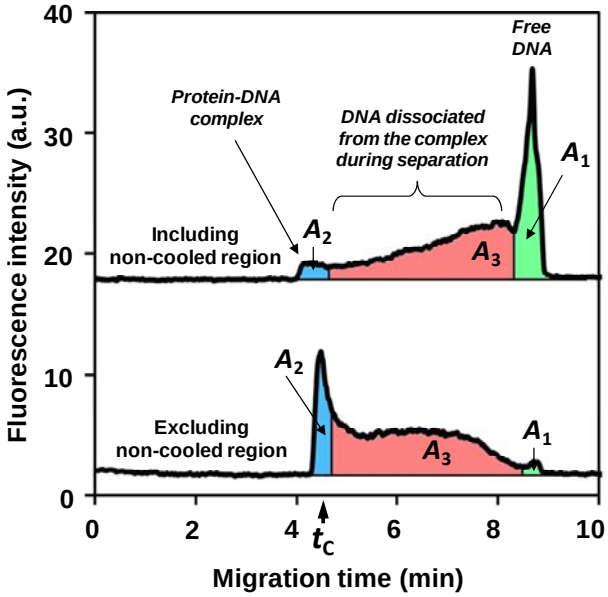

Figure S1. Illustration of NECEEM electropherograms showing all the parameters needed for the determination of K_d and k_{off}. The electropherograms are identical to those in Fig. 2 of the main text.

2. Determination of DNA Concentration by DNA Hybridization Analysis

The concentration of the DNA target was calculated using the following equation:

$$[\text{Target}] = [\text{Probe}]\frac{A_2}{(A_1 + A_2)} \qquad \textbf{(S3)}$$

where [Target] is the unknown concentration of the target and [Probe] is a known concentration of the probe and A_1 and A_2 are the areas corresponding to the peaks of unbound probe and bound probe as illustrated in **Fig. S2**. Not to complicate the matter, the calculations do not include the relative quantum yield of fluorescence of the target-bound probe (see reference 21 in main text).

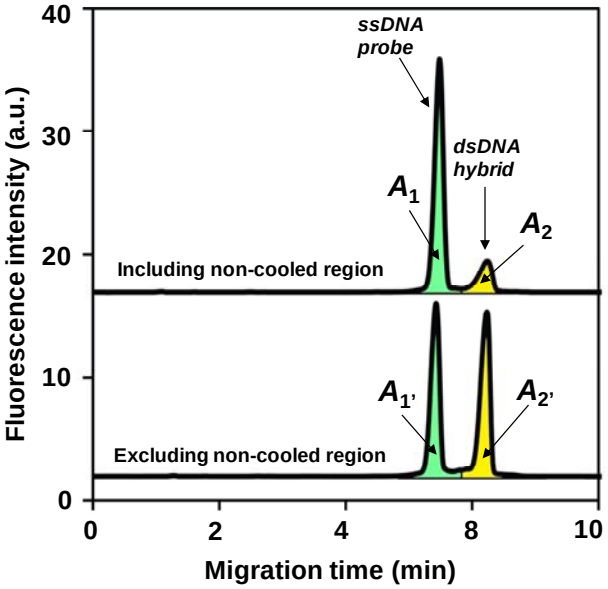

Figure S2. Illustration of electropherograms obtained in a CE-based DNA hybridization assay showing the areas (colored) required for the determination of unknown target concentration. The electropherograms are identical to those in Fig. 4 of the main text.