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Making DNA Hybridization Assays in Capillary Electrophoresis Quantitative

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A single-stranded DNA-binding protein (SSB) has been recently proven to facilitate highly efficient separation of the excess hybridization probe from the probe–target hybrid in gel-free capillary electrophoresis. SSB added to the electrophoresis run buffer binds the single-stranded DNA probe but does not bind the double stranded DNA–DNA or DNA–RNA hybrid. As a result, SSB changes the electrophoretic mobility of the probe but does not affect the mobility of the hybrid. If the probe is labeled fluorescently, real-time sensitive detection can be facilitated. While the concept of SSB-mediated hybridization analysis has been proven in principle, the question of how to make such analysis quantitative without building a calibration curve remains open. Here, we propose a general approach for making SSB-mediated analysis quantitative. This approach takes into account such phenomena as (i) the potential influence of the probe–target hybridization and probe–SSB binding on the quantum yield of the fluorescent label and (ii) the potential dissociation of the hybrid by SSB. The proposed approach was used to study the quenching and the dissociation phenomena experimentally. We proved for the first time that SSB does not detectably dissociate the probe–target hybrid, which significantly simplifies the analysis.

Hybridization assays, which detect DNA or RNA targets using labeled DNA probes with sequences complementary to those of the targets, are a key tool in molecular biology.[1–3] Figure 1 shows the general concept of such assays. In step 1, the excess of a labeled probe (P*) is added to a target (T)-containing sample to bind the majority of the target and have only the TP hybrid and unreacted P left. In step 2, TP* is separated from P* to facilitate the quantitation of TP* using the label on P* for detection. The most well-known examples of hybridization assays are Southern and Northern blotting analyses in which the separation step is achieved on a surface.[4,5] The target is photolinked on a nylon membrane prior to the hybridization step. Accordingly, the hybrid is also linked while the unreacted probe is not bound and can be removed by rigorously washing the membrane with a probe-free buffer. Southern and Northern analyses are time-consuming procedures (take no less than 24 h) with a relatively high limit of detection (10–50 μg of DNA or RNA) despite using radioactive labels, which impose potential hazard.[6] In addition, these analyses are semiquantitative unless calibration is performed with known amounts of a specific target with which one needs to work.

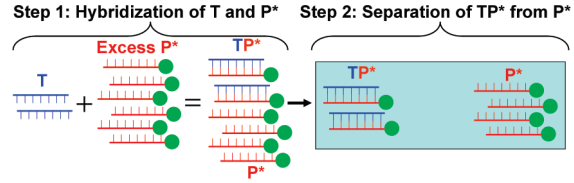

Figure 1. Schematic illustration of hybridization analysis. In step 1, the target sequence T is hybridized with a labeled probe P* to form the target–probe hybrid, TP*, through mixing the target with the excess of the probe. In step 2, the unreacted probe is separated from the target–probe hybrid to facilitate the quantitation of the hybrid.

Ideally, in a hybridization assay, the fast hybridization reaction between the target and nonradioactively labeled probe would be followed by their efficient and fast separation, which in turn, would be accompanied by real-time quantitation of both the hybrid and the unbound probe. Capillary electrophoresis (CE) provides an instrumental platform for fast hybridization assays with real-time detection. Some CE-based hybridization assays have been developed including gel and gel-free ones.[7–10] One of the gel-free hybridization analyses is based on separation of the unbound probe from the target–probe hybrid by affinity capillary electrophoresis mediated by single-stranded DNA binding protein (SSB). The significant mobility shift of DNA by binding to SSB in CE was demonstrated in 2000 by Wan and Le.[11] In the hybridization analysis based on this shift, both the probe and the hybrid were quantified with a fluorescent label on the probe.[7,8] In this approach, SSB is added to the electrophoresis run buffer where it binds the single-stranded probe but does not bind the double-

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stranded hybrid. By binding the probe, SSB drags ssDNA away from the hybrid, whose mobility is not affected by SSB (see Figure S1 in Supporting Information). If the probe is labeled fluorescently, real-time sensitive detection can be facilitated. While the concept of SSB-mediated hybridization analysis has been experimentally demonstrated relatively a long time ago,[7,8] the question of how to make such analysis quantitative remains open. To address this question, here we propose a general approach for making SSB-mediated analysis quantitative. The quantity of the target is determined taking into consideration: (i) the potential influence of the probe−target hybridization and probe−SSB binding on the quantum yield of the fluorescent label and (ii) potential dissociation of the hybrid by SSB. The proposed approach was used to study the quenching and the dissociation phenomena for an experimental model which included a 22-nt long ssDNA target and the same length fluorescently labeled DNA probe. We showed that the binding of the probe to either the target or SSB reduces the quantum yield of fluorescence. On the other hand, SSB was proved not to detectably dissociate the hybrid. Finally, we proved that when the probe quenching is taken into account, the SSB-mediated hybridization analysis is quantitative without the requirement of building a calibration curve.

RESULTS AND DISCUSSION

Theoretical Consideration. *Target Amount.* We assume that the fluorescently labeled probe, P* (asterisk designates the label), hybridizes with the target T, in a process that goes to completion:

$$T + P^* \rightarrow TP^* \tag{1}$$

where TP* is the target−probe hybrid. We also assume that the probe is taken in excess to the target. In this case, the initial amounts of the probe and target, P^*_0 and T_0, are linked with the amounts of the remaining probe, P*, and formed hybrid, TP*, as:

$$P^*_0 = TP^* + P^* \tag{2}$$

$$T_0 = TP^* \tag{3}$$

where the amounts can be expressed in any conventional units such as moles or number of molecules. In the rest of this work, symbols A with corresponding indexes denote measurable areas of peaks in electropherograms, which are equivalent to integrated fluorescent signals and are presented in arbitrary units. To compensate for differences in the residence time in the detector, these areas should be divided by corresponding migration times if on-column detection is used. To be proportional to the amount of the fluorophore, the areas should be divided by the absolute quantum yield of fluorescence, Q.

Using eq 2, we can express the known initial amount of probe through: (i) fluorescence signals from the hybrid, A_{TP^*}, and remaining free probe, A_{P^*}, (ii) corresponding absolute quantum yields of the target−bound probe, Q_{TP^*}, and free probe, Q_{P^*}, and (iii) a proportionality coefficient a, which is constant for the same fluorophore and the same detector:

$$P^*_0 = a(A_{TP^*}/Q_{TP^*} + A_{P^*}/Q_{P^*}) \tag{4}$$

The quantum yields are unitless, and the coefficient a has units of amount (moles or number of molecules).

Using eq 3, we can express the sought initial amount of the target through A_{TP^*}, Q_{TP^*}, and a:

$$T_0 = aA_{TP^*}/Q_{TP^*} \tag{5}$$

To eliminate the unknown coefficient a, we divide eq 5 by eq 4 and obtain the following equation for finding the unknown amount of the target:

$$T_0 = P^*_0 \frac{A_{TP^*}/Q_{TP^*}}{A_{TP^*}/Q_{TP^*} + A_{P^*}/Q_{P^*}} \tag{6}$$

To simplify the analysis, both the numerator and denominator in eq 6 are multiplied by Q_{P^*} to lead to:

$$T_0 = P^*_0 \frac{A_{TP^*}/q_{TP^*}}{A_{TP^*}/q_{TP^*} + A_{P^*}} \tag{7}$$

where q_{TP^*} is the relative quantum yield of the target−bound probe with respect to that of the free probe: $q_{TP^*} = Q_{TP^*}/Q_{P^*}$.

We will now consider the case of SSB being present in the run buffer under an assumption of fast equilibration between SSB−bound probe and SSB−unbound probe. In the presence of SSB, a fraction, f, of target−unbound probe is bound to SSB and another fraction, $1 - f$, is SSB−unbound probe. The effective relative quantum yield of the probe "fractionally" bound to SSB, $q^{SSB}_{P^*}$, is a sum of two terms corresponding to the SSB−unbound and SSB−bound fractions:

$$q^{SSB}_{P^*} = q_{P^*}(1 - f) + q_{SSB\cdot P^*}f = 1 - f(1 - q_{SSB\cdot P^*}) \tag{8}$$

where the relative quantum yield of the unbound probe, q_{P^*}, is equal to one because we define all quantum yields relative to that of unbound probe and the relative quantum yield of the SSB−bound probe, $q_{SSB\cdot P^*}$, will be less than 1 if SSB quenches fluorescence. To compensate for the potential quenching of fluorescence by SSB, the fluorescence signal of the probe in the presence of SSB, $A_{P^*}{}^{SSB}$, should be divided by the effective quantum yield defined in eq 8, and accordingly, eq 7 will have the form:

$$T^{SSB}_0 = P^*_0 \frac{A_{TP^*}/q_{TP^*}}{A_{TP^*}/q_{TP^*} + A^{SSB}_{P^*}/q^{SSB}_{P^*}} \tag{9}$$

In addition to quenching probe fluorescence, SSB may potentially destabilize the hybrid and promote its dissociation into free probe and target. In general, the target−probe hybrid is typically very stable at nondenaturing conditions. However, SSB can potentially bind the stable hybrid with low but finite affinity and potentially break the complex by competitively replacing one of the strands in the hybrid. Although, to the best of our knowledge, the effect of SSB on hybrid stability has never been investigated, it is conceivable to suggest that such an effect can be significant under certain conditions, such as higher temperature, shorter probe, low salt concentration, etc. Therefore, in our theoretical

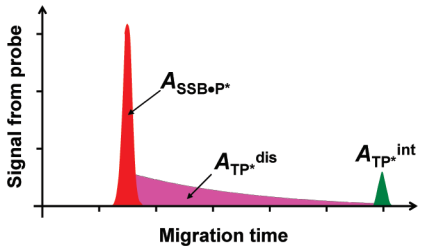

Figure 2. Conceptual illustration of a NECEEM electropherogram if SSB present in the electrophoresis run buffer dissociates the hybrid, TP*, into free probe, P*, and free target, T. Area $A_{SSB\cdot P*}$ corresponds to the SSB-bound excess of probe. Area A_{TP*}^{int} corresponds to the target−probe hybrid intact by the time of reaching the detector. A_{TP*}^{dis} corresponds to the target−probe hybrid dissociated by SSB during electrophoresis and bound to SSB after that.

consideration, we take the potential effect of SSB on complex stability into account.

If SSB, which is present in the run buffer, accelerates hybrid dissociation, then such accelerated dissociation will be a continuous process during the entire separation. The dissociated probe will be bound by SSB and will start migrating with a velocity different from that of the hybrid. It will be separated from the free target and will be unable to reform the hybrid. Hybrid dissociation will follow the monomolecular decay pattern with a pseudofirst-order rate constant k, which is a function of concentration of SSB:

$$TP* \xrightarrow{k \sim [SSB]} T + P* \tag{10}$$

This kind of separation is termed nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM).[12] If hybrid dissociation is significant, the area of the intact hybrid in the NECEEM electropherogram will decrease and an exponential decay area between the peaks of the hybrid and SSB-shifted probe will appear (Figure 2). The probe formed by hybrid dissociation is bound to SSB with the same fraction coefficient f that characterizes binding of free probe to SSB (see above). The same f allows us to use for this area the same effective quantum yield q_{P*}^{SSB} defined in eq 8. In order to generalize eq 9 for the case of hybrid dissociation, we should replace the term A_{TP*}/q_{TP*} with a sum of two terms corresponding to the remaining intact hybrid, A_{TP*}^{int}/q_{TP*}, and the dissociated hybrid, $A_{TP*}^{dis}/q_{P*}^{SSB}$:

$$T_0^{SSB} = P_0^* \frac{A_{TP*}^{int}/q_{TP*} + A_{TP*}^{dis}/q_{P*}^{SSB}}{A_{TP*}^{int}/q_{TP*} + (A_{TP*}^{dis} + A_{P*})/q_{P*}^{SSB}} \tag{11}$$

If SSB-induced hybrid dissociation is significant (that is if A_{TP*}^{int}/q_{TP*} is not much greater than $A_{TP*}^{dis}/q_{P*}^{SSB}$), then eq 11 should be used instead of eq 9 to calculate the unknown amount of the target in the hybridization analysis.

Fraction of Bound Probe. The fraction of SSB−bound probe can be found (see Supporting Information) using the apparent migration time of the probe, t, for the concentration of SSB chosen for the assay as well as the migration times of the SSB−unbound probe, t_{P*}, and SSB−bound probe, $t_{SSB\cdot P*}$:

$$f = \frac{t_{SSB\cdot P*}}{t} \frac{t_{P*} - t}{t_{P*} - t_{SSB\cdot P*}} \tag{12}$$

The value of $t_{SSB\cdot P*}$ can be measured experimentally at a saturating concentration of SSB. The saturating concentration of SSB should be higher than the equilibrium dissociation constant, K_d, of SSB−probe complex, thus ensuring that most of the probe is SSB−bound.

Quantum Yields. Assuming that the probe can be baseline separated from the hybrid in the absence of SSB, the relative quantum yields of the target−bound probe, q_{TP*}, can be found from two sets of data obtained for the same amount of the probe with and without the target, respectively:

$$q_{TP*} = \frac{A_{TP*}}{A_{P*}^{[T]=0} - A_{P*}^{[T]\neq 0}} \tag{13}$$

where $A_{P*}^{[T]=0}$ is the fluorescent signal of the probe for no target and $A_{P*}^{[T]\neq 0}$ and A_{TP*} are fluorescent signals of the remaining target−unbound probe and target−bound probe, respectively, for a nonzero target concentration.

The relative quantum yield of SSB−bound probe, $q_{SSB\cdot P*}$, can be found from two areas of target−unbound probe obtained in the absence of SSB, A_{P*}, and at a saturating concentration of SSB, $A_{P*}^{[SSB]_{sat}}$:

$$q_{SSB\cdot P*} = \frac{A_{P*}^{[SSB]_{sat}}}{A_{P*}} \tag{14}$$

The above theoretical consideration was then tested experimentally.

EXPERIMENTAL RESULTS

Experimental Model. To demonstrate the applicability of our quantitative approach to SSB-mediated hybridization analysis, we used SSB from *E. coli* which is known to bind a minimum of 8-mer ssDNA. A 22-mer DNA of a random sequence was used as a target and a 22-mer DNA with a sequence complementary to that of the target was used as a probe. The probe was labeled with Alexa Fluor 488 dye through an ester linker at the 5′ end. An internal standard, fluorescein, was added to the 100 nM DNA probe solution (50 mM Tris−acetate, 50 mM NaCl, 10 mM EDTA, pH 8.2), and all the results were normalized with respect to its peak area to compensate for variations in the injected volume. Another phenomenon that could potentially influence quantitative results is photobleaching of the fluorescent label on the probe. Indeed, since the migration times of the probe and hybrid are different, their residence times in an on-capillary detector are also different and the label on the probe and hybrid could photobleach to different extents. In such a case, the degree of photobleaching would need to be included as a parameter in quantitation. (Note that photobleaching of the internal standard cannot influence quantitative results since its migration and residence times do not change.) The extent of photobleaching can be measured using a repetitive passage approach; and photobleaching can be taken into account by including a photobleaching coefficient in the calculations.[13] In this work, Alexa Fluor 488 dye, which does not

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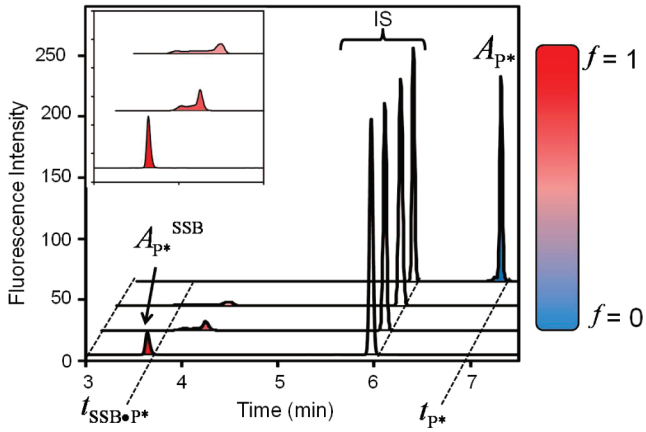

Figure 3. Quenching of probe, P*, fluorescence upon binding to SSB. The study was conducted by CE with the run buffer containing SSB at varying concentrations: in descending order 0 (top trace), 5, 10, and 50 nM (bottom trace). The injected amount of the probe was 27×10^{-16} mol; 100 nM in an injected plug of 27 nL. The inset shows the peaks corresponding to the SSB−bound probe enlarged in the vertical direction. The internal standard (IS) was 100 nM fluorescein.

detectably photobleach during a passage through the detection zone, was used as a label to exclude photobleaching from our quantitative consideration.

Binding of the Probe to SSB. The influence of SSB on probe migration and fluorescence was studied by varying the concentration of SSB in the run buffer. We found that with increasing concentration of SSB the peak of the probe was shifting to the left and reaching its leftmost position when the concentration of SSB exceeded 50 nM (Figure 3). At these high concentrations, the fraction of SSB−bound probe was approaching unity. For our set of conditions, we defined the saturating concentration of SSB as $[SSB]_{sat} = 50$ nM. It should be noted that the decrease of the fluorescence peak in the presence of SSB could not be attributed to the binding of the SSB−DNA complex to capillary walls as no fluorescence material was washed from the capillary after separation.

If the working concentration of SSB chosen for the hybridization assay is below the saturating one, then the fraction of SSB−bound probe is not equal to unity and it should be calculated using eq 12 with experimentally determined times. As an example, we calculated the fraction of SSB−bound probe for [SSB] = 10 nM using the time data in Figure 3; the fraction was $f \sim 0.6$. The non-Gaussian shape of the peak for the nonsaturating [SSB] is likely caused by the relatively slow equilibration between the probe and SSB−bound probe in the following reaction:

$$SSB + P^* \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} SSB \cdot P^* \qquad (15)$$

Indeed, the peak shape depends on the relation between the characteristic equilibration time in reaction 15, t_{eq}, and the characteristic separation time, t_{sep}, of SSB·P* and P*. If the concentration of SSB is greater than that of the probe, the characteristic time of equilibration can be accurately derived, and it is governed by the following expression (see Supporting Information):

$$t_{eq} = 1/(k_{on}[SSB] + k_{off}) \qquad (16)$$

The characteristic time of separation of SSB·P* from P* is the time required for zones of SSB·P* and P* to separate from each other:

$$t_{sep} = w/|v_{SSB \cdot P^*} - v_{P^*}| \qquad (17)$$

where w is the width of the initial zone of the probe. In general, if $t_{eq} > t_{sep}$, the zones of SSB·P* and P* will be separated before re-equilibration in reaction 15 proceeds to a significant extent. Thus, SSB·P* and P* will be moving as separate zones and will generate two peaks. If $t_{eq} \sim t_{sep}$, re-equilibration in reaction 15 and separation proceed with comparable rates. As a result, SSB·P* and P* will be moving as two zones with a "bridge" between them. Finally, if $t_{eq} < t_{sep}$, the re-equilibration in reaction 15 occurs much faster than zone separation, and as a result, SSB·P* and P* will be moving as a single zone. This final scenario is preferable for the SSB-mediated hybridization analysis and can be achieved by minimizing t_{eq} through increasing [SSB] to at least $[SSB]_{sat}$.

To determine the quantum yield, $q_{SSB \cdot P^*}$, of SSB−bound probe, we increased the concentration of SSB to the saturating one (see Figure 3). The fraction of SSB−bound probe was equal to unity, and the quantum yield could be determined with eq 14 using the two areas, $A_{P^*}^{[SSB]_{sat}}$ and A_{P^*}, found from Figure 3. Our calculations showed that this quantum yield was $q_{SSB \cdot P^*} = 0.24 \pm 0.02$. (The average and the standard deviation were calculated based on 20 experiments.)

It should be emphasized that the quantum yields measured depend on the nature of the probe and target as well as the buffer composition. Therefore, the quantum yields have to be measured for every new set of conditions.

Binding of the Probe to the Target. The influence of the target on probe migration and fluorescence was studied by hybridizing the target to the excess of the probe. The amounts of the target and probe were chosen such that the areas of their corresponding peaks in CE would be comparable and could be accurately measured. To study the influence of the target on probe fluorescence in SSB-free solution, P* should be baseline separated from TP*. While such separation is challenging, we were able to achieve it through carefully selecting the separation conditions (Figure 4). It should be noted that, while the resolution of SSB-free separation, $R_S^{[SSB]=0} = 7.2$, is suitable for our goal of calculating the quantum yield, it is much less than that of SSB-mediated separation, $R_S^{[SSB]_{sat}} = 35.6$. To calculate the quantum yield of the target−bound probe, q_{TP^*}, we conducted two CE experiments with two amounts of the target, one of which was zero, and the same amount of the probe. From CE electropherograms presented in Figure 4, we determined the three areas, $A_{P^*}^{[T]=0}$, $A_{P^*}^{[T]\neq 0}$, and A_{TP^*}, required for the calculation of the quantum yield of the target−bound probe using eq 13. We found that this quantum yield was also significantly different from unity: $q_{TP^*} = 0.61 \pm 0.02$. (The average and the standard deviation were calculated based on 24 experiments.) The difference from unity quantum yields require that eq 9 be used for finding T_0.

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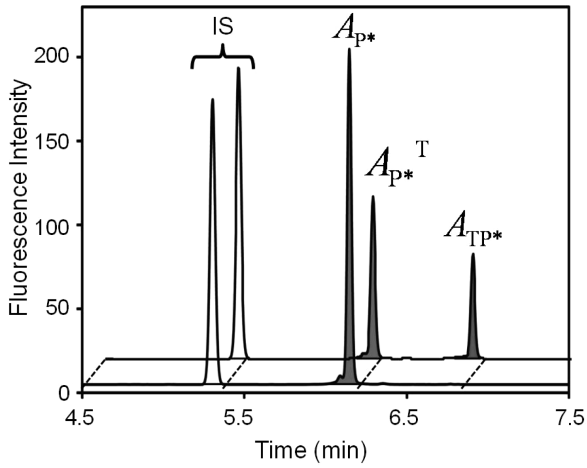

Figure 4. Quenching of probe, P*, fluorescence upon its binding to the target, T. The study was conducted with CE by injecting identical 27 nL samples containing identical amounts of the probe, 27×10^{-16} mol, but different amounts of the target: 0 (bottom trace) and 6.75×10^{-16} mol (top trace). The concentration of the probe was 100 nM while the concentrations of the target were 0 and 25 nM for the bottom and top traces, respectively. The internal standard (IS) was 100 nM fluorescein.

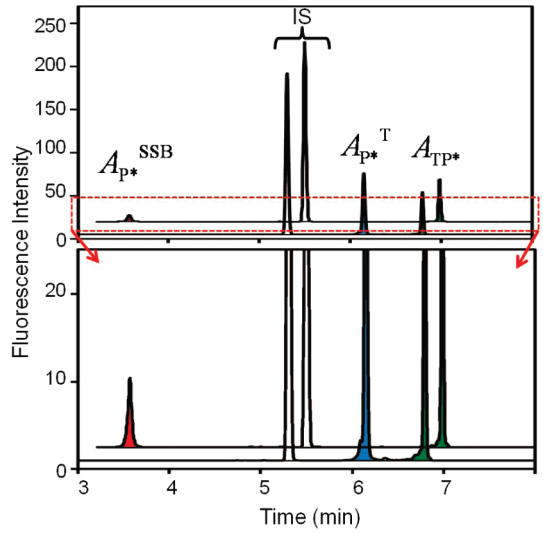

Figure 5. Influence of SSB on the hybridization assay conducted by CE. The injected 27 nL sample contained 27×10^{-16} mol of the probe, P*, and 6.75×10^{-16} mol of the target, T, resulting in final concentrations of 100 and 25 nM for the probe and target, respectively. The concentration of SSB in the run buffer was either 0 (bottom trace) or 50 nM (top trace). The bottom panel is identical to the top one except for a different scale of the *y*-axis. The internal standard (IS) was 100 nM fluorescein.

Influence of SSB on Hybrid Stability. To answer the question of whether or not SSB causes target dissociation, we conducted an experiment identical to those used in an actual hybridization analysis. The target−probe mixture with an excess of the probe was injected into the capillary and run in an electrophoresis buffer containing the saturating concentration of SSB. The obtained electropherograms (Figure 5) show no detectable difference between the A_{TP*} and A_{TP*}^{SSB} (within the 11% experimental error of area calculation) and, accordingly, no detectable signal from the dissociated hybrid. These results suggest that SSB does not appreciably bind and dissociate the hybrid under our conditions. As a result, eq 9 can be used instead of eq 11 for finding the unknown amount of the target.

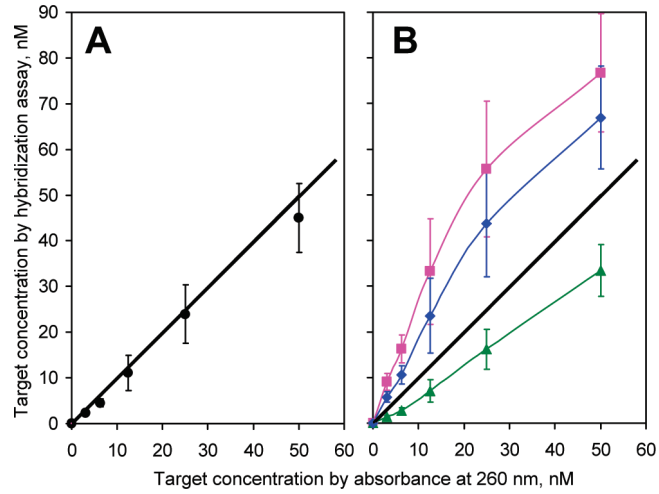

Figure 6. Target recovery in SSB-mediated hybridization assay with different ways of correction for quenching of probe fluorescence. Panel A: $q_{SSB \cdot P*} = 0.24$ and $q_{TP*} = 0.61$. Panel B: $q_{SSB \cdot P*} = 1$ and $q_{TP*} = 0.61$ (squares), $q_{SSB \cdot P*} = 0.24$ and $q_{TP*} = 1$ (triangles), and $q_{SSB \cdot P*} = q_{TP*} = 1$ (diamonds). The black line is a theoretically expected dependence. Each experimental point is an average and standard deviation obtained from 4 experiments. The concentrations of probe and SSB were 100 and 50 nM, respectively, in all experiments.

Quantitative Calibration-Free Hybridization Analysis. To demonstrate the correctness of the approach developed above, we conducted a hybridization analysis with a constant 100 nM concentration of the probe and target concentrations varying within 0−50 nM. The saturating concentration of SSB was used to ensure that $f = 1$. The areas determined from the electropherograms (Figure S2, Supporting Information) and the quantum yields, $q_{TP*} = 0.61 \pm 0.02$ and $q_{SSB \cdot P*} = 0.24 \pm 0.02$, determined above were used for calculations of T_0 with eq 9. We found that the calculated amounts were identical to the loaded amounts measured spectrophotometrically within the limits of experimental error (Figure 6A), thus confirming that the analysis was quantitative without building a calibration curve. The relative standard deviations of the quantum yields were 2 and 8%, respectively. They were small in comparison to the errors in target concentrations, which were caused by a cumulative influence of multiple parameters. Therefore, when calculating target concentrations for Figure 6, we used average values of the quantum yields and disregarded the contribution of their small errors to the experimental error of the calculated concentrations. The four experiments that Figure 6 is based on were performed on 3 days over a period of 3 weeks. Thus, the day-to-day variation is incorporated in the errors shown in Figure 6.

To demonstrate the inaccuracies that could be caused by ignoring the quenching of fluorescence, it is instructive to calculate the target amount for three assumptions: (i) no quenching by SSB ($q_{SSB \cdot P*} = 1$), (ii) no quenching by T ($q_{TP*} = 1$), and (iii) no quenching by both SSB and T ($q_{SSB \cdot P*} = q_{TP*} = 1$). The results plotted in Figure 6B show that the deviation of the incorrectly calculated T_0 from the loaded T_0 is as high as 2.5 times.

To summarize, while developing an SSB-mediated quantitative hybridization assay for specific target, probe, and SSB, one needs

to measure the relative quantum yields of the hybrid, q_{TP^*}, and SSB−bound probe, $q_{SSB\cdot P^*}$, and determine whether or not SSB dissociates the hybrid. If SSB dissociates the hybrid, the most comprehensive eq 11 should be used; otherwise, eq 9 is sufficient. It is also essential to find and use the saturating concentration of SSB which guarantees that most of the target−unbound probe is bound to SSB. While eqs 9 and 11 operate with amounts of the probe and target, the amounts can be simply replaced with concentrations.

CONCLUSIONS

SSB in the electrophoresis run buffer dramatically widens the separation window between the probe−target hybrid and SSB−bound excess of the probe. The wide separation window has an important advantage. The large window allows us to use a large excess of the probe to the target without the interference between the corresponding peaks of the probe and hybrid. Using a high concentration of the probe is beneficial as it shortens the hybridization time and can be used for a wider range of target concentrations. With the approach developed in this work, the SSB-mediated hybridization analysis can now be made quantitative without building a calibration curve. Since this manuscript was submitted, we measured the quantum yields of two more probes one of which was 37-nt long DNA labeled with Alexa Fluor 488 and the second one was 23-nt long DNA labeled with 6-carboxyfluorescein (both at the 5′ end). The quantum yields were found to be $q_{TP^*} = 0.59 \pm 0.11$ and $q_{SSB\cdot P^*} = 0.12 \pm 0.01$ for the 37-long probe and $q_{TP^*} = 0.20 \pm 0.06$ and $q_{SSB\cdot P^*} = 0.34 \pm 0.04$ for the 23-nt long probe. While the nature of this large variation requires further detailed studies, the variation itself emphasizes the need for accurate measurements of the quantum yields.

MATERIALS AND METHODS

Materials. All DNA for hybridization assays were custom-synthesized by IDT (Coralville, IA). The hybridization probe was 5′-(Alexa Fluor 488)-TCACAAGTTAGGGTCTCAGGGA-3′. The hybridization target was 5′-TCCCTGAGACCCTAACTTGTGA-3′. SSB (single-stranded DNA binding protein), from *E. coli* was from Epicentre Biotechnologies (Madison, WI). All other materials were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

Hybridization Conditions. All hybridizations were carried out in a Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Varying concentrations of DNA probe and DNA target were incubated with 100 nM fluorescein in the incubation buffer (50 mM Tris-acetate, 50 mM NaCl, 10 mM EDTA, pH 8.2). Temperature was increased to a denaturing 80 °C and lowered to 20 °C in decremental steps of 1 °C every 3 s to allow annealing.

Spectrophotometric Determination of Target Concentration. DNA target concentration was determined by light absorption at 260 nm using the Nano-Drop ND-1000 spectrophotometer (Thermo-Fisher Scientific). The stock concentration of the target was too high to measure directly; therefore, a small sample of the stock solution was serially diluted, and absorbance of each sample at 260 nm was measured 3 times. The concentration of each sample was determined as $absorbance/\varepsilon l$ where ε is the molar extinction coefficient of the DNA target at 260 nm (provided by IDT) and l is the optical path length. Using the concentrations of the serial dilutions, the original stock concentration was extrapolated.

CE. A P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA) was used with laser-induced fluorescence detection. The fluorescence was excited with a 488 nm line argon-ion laser. Uncoated fused-silica capillaries were used with an inner diameter of 75 μm and an outer diameter of 365 μm. The total length of the capillary was 50 cm with a distance of 40 cm to the detector. The running buffer was 25 mM sodium tetraborate, pH 9.3, with or without 50 nM SSB. The capillary was rinsed prior to each run with a sequence of 100 mM HCl, 100 mM NaOH, double-distilled water, and 25 mM sodium tetraborate, pH 9.3 for 1 min each. Samples were injected by a pressure pulse of 0.5 psi for 5 s; the volume of the injected sample plug was ~27 nL. Electrophoresis was driven by an electric field of 500 V/cm. The capillary temperature was maintained at 20 °C. After runs, the capillary was washed with the fluorescence detector turned on to monitor potential adsorption of fluorescently labeled DNA to capillary walls.

Prior to being used in calculations, the peak areas of the hybridized probe (A_{TP^*}) and free probe in the presence of SSB ($A_{P^*}^{SSB}$) were normalized by dividing their measured values by their respective migration times (t_{TP^*} and $t_{P^*}^{SSB}$) as well as by the peak area of the internal standard (A_{IS}) also normalized by its migration time (t_{IS}):

$$A_{TP^*}^{\text{normalized}} = \frac{A_{TP^*}}{t_{TP^*}}\frac{t_{IS}}{A_{IS}}$$

$$A_{P^*}^{\text{SSBnormalized}} = \frac{A_{P^*}^{SSB}}{t_{P^*}^{SSB}}\frac{t_{IS}}{A_{IS}}$$

For simplicity, the word “normalized” is omitted in the manuscript.

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SUPPORTING INFORMATION AVAILABLE

Supporting mathematical appendix and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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SUPPORTING INFORMATION

Making DNA Hybridization Assays in Capillary Electrophoresis Quantitative

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MATHEMATICAL APPENDIX

1. Fraction of SSB-bound probe as a function of migration times

Velocity is an additive function, therefore, the apparent velocity of the probe, v, in electrophoresis with SSB present in the run buffer (assuming fast equilibrium between free probe and SSB-bound probe) a sum of two terms contributed by free probe, P, and SSB-bound probe, SSB•P*:

$$v = v_{\mathrm{P}^*} f_{\mathrm{P}^*} + v_{\mathrm{SSB}\bullet\mathrm{P}^*} f_{\mathrm{SSB}\bullet\mathrm{P}^*} \tag{1.1}$$

where v_{P} and $v_{\mathrm{P}\bullet\mathrm{SSB}}$ are velocities of free and SSB-bound probe and f_{P^*} and $f_{\mathrm{SSB}\bullet\mathrm{P}^*}$ are fractions of free and SSB-bound probe. The fractions are defined through concentrations as:

$$f_{\mathrm{P}^*} = \frac{[\mathrm{P}^*]}{[\mathrm{P}^*]_0}, \quad f_{\mathrm{SSB}\bullet\mathrm{P}^*} = \frac{[\mathrm{SSB}\bullet\mathrm{P}^*]}{[\mathrm{P}^*]_0} \tag{1.2}$$

$$[\mathrm{P}^*]_0 = [\mathrm{P}^*] + [\mathrm{SSB}\bullet\mathrm{P}^*]$$

A sum of the fractions is equal to unity:

$$f_{\mathrm{P}^*} + f_{\mathrm{SSB}\bullet\mathrm{P}^*} = 1 \tag{1.3}$$

Using equation (1.3), we can rewrite equation (1.1) as:

$$v = v_{\mathrm{P}^*}(1 - f) + v_{\mathrm{SSB}\bullet\mathrm{P}^*} f \tag{1.4}$$

And express the fraction of bound probe through the velocities:

$$f = \frac{v - v_{\mathrm{P}^*}}{v_{\mathrm{SSB}\bullet\mathrm{P}^*} - v_{\mathrm{P}^*}} \tag{1.5}$$

It is more convenient to work with experimentally measured migration times, t, than with velocities. The velocity is reciprically proportional to migration times:

$$v = L/t, \quad v_{\mathrm{P}^*} = L/t_{\mathrm{P}^*}, \quad v_{\mathrm{SSB}\bullet\mathrm{P}^*} = L/t_{\mathrm{SSB}\bullet\mathrm{P}^*} \tag{1.6}$$

where L is the capillary length to the detector. Using equations (1.5) and (1.6) we ca express the fraction of the SSB-bound probe through the migration times:

$$f = \frac{1/t - 1/t_{\mathrm{P}^*}}{1/t_{\mathrm{SSB}\bullet\mathrm{P}^*} - 1/t_{\mathrm{P}^*}} = \frac{t_{\mathrm{SSB}\bullet\mathrm{P}^*}}{t} \frac{t_{\mathrm{P}^*} - t}{t_{\mathrm{P}^*} - t_{\mathrm{SSB}\bullet\mathrm{P}^*}} \tag{1.7}$$

2. Equilibration time

The equilibration time, t_{eq}, can be precisely found for a pseudo-first order reaction of probe, P*, binding to SSB. Let's assume that the concentration of SSB is greater than that of the probe: [SSB] >> [P*]. In our case of SSB present in the run buffer, this condition can be reduced to [SSB] ≥ [P*]. Indeed, since SSB fills the entire length of the capillary and P* fills only a short part of it, even if the local concentration of SSB in the vicinity of P* is reduced due to binding to

P*, the remaining SSB-unbound P* rapidly moves to a new volume with "untouched" SSB. We can thus assume that [SSB] = const even if [SSB] ≥ [P*].

Our goal is to find the characteristic equilibration time, t_{eq}, of the following reaction:

$$\mathrm{SSB} + \mathrm{P^*} \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} \mathrm{SSB \bullet P^*} \qquad (2.1)$$

where k_{on} is the rate constant of complex formation and k_{off} is the rate constant of complex dissociation. By definition, the characteristic equilibration time is reciprocal of the observed rate constant of changing either [P*] or [SSB•P*]:

$$t_{eq} = 1/k_{obs} \qquad (2.2)$$

Using the pseudo-first order approximation we can assume that:

$$k_{on}[\mathrm{SSB}] = \mathrm{const} = k_{on}^{app} \qquad (2.3)$$

We can then write for the rate of the reaction (2.1):

$$\text{Rate of reaction} = \frac{d[\mathrm{SSB \bullet P^*}]}{dt} = -\frac{d[\mathrm{SSB}]}{dt} = -\frac{d[\mathrm{P^*}]}{dt} = \\ = k_{on}[\mathrm{SSB}][\mathrm{P^*}] - k_{off}[\mathrm{SSB \bullet P^*}] = k_{on}^{app}[\mathrm{P^*}] - k_{off}[\mathrm{SSB \bullet P^*}] \qquad (2.4)$$

Lets solve equation (2.4) for d[SSB•P*]/dt and for the initial concentration of SSB•P* equal to zero and initial concentration of P* equal to $[\mathrm{P^*}]_0$. Mass balance requires that:

$$[\mathrm{P^*}] + [\mathrm{SSB \bullet P^*}] = [\mathrm{P^*}]_0 \qquad (2.5)$$

Using equation (2.5) we can write for the rate of reaction:

$$\frac{d[\mathrm{SSB \bullet P^*}]}{dt} = k_{on}^{app}[\mathrm{P^*}] - k_{off}[\mathrm{SSB \bullet P^*}] = \\ = k_{on}^{app}\left([\mathrm{P^*}]_0 - [\mathrm{SSB \bullet P^*}]\right) - k_{off}[\mathrm{SSB \bullet P^*}] = \\ = k_{on}^{app}[\mathrm{P^*}]_0 - k_{on}^{app}[\mathrm{SSB \bullet P^*}] - k_{off}[\mathrm{SSB \bullet P^*}] = \\ = k_{on}^{app}[\mathrm{P^*}]_0 - (k_{on}^{app} + k_{off})[\mathrm{SSB \bullet P^*}] \qquad (2.6)$$

From equation (2.6) we can obtain an ordinary differential equation with separated variables [SSB•P*] and t:

$$\frac{d[\mathrm{SSB \bullet P^*}]}{k_{on}^{app}[\mathrm{P^*}]_0 - (k_{on}^{app} + k_{off})[\mathrm{SSB \bullet P^*}]} = dt \qquad (2.7)$$

Equation (2.7) can be directly integrated using the following tabular intergal:

$$\int \frac{dx}{ax + b} = \int dt \qquad (2.8)$$

The solution of equation (2.7) is:

$$[\mathrm{SSB \bullet P^*}] = \frac{k_{on}[\mathrm{SSB}][\mathrm{P^*}]_0}{k_{on}[\mathrm{SSB}] + k_{off}}\left\{1 - e^{-(k_{on}[\mathrm{SSB}] + k_{off})t}\right\} = \frac{k_{on}[\mathrm{SSB}][\mathrm{P^*}]_0}{k_{obs}}\left\{1 - e^{-k_{obs}t}\right\} \qquad (2.9)$$

From equation (2.9) we can find the observed rate constant, k_{obs}:

$$k_{obs} = k_{on}[\mathrm{SSB}] + k_{off} \qquad (2.10)$$

Finally, an expression for the characteristic equilibration time is:

$$t_{eq} = 1/k_{obs} = 1/\left(k_{on}[\mathrm{SSB}]_0 + k_{off}\right) \qquad (2.11)$$

Equation (2.11) governs the equilibration between SSB-bound and SSB-unbound probe under an assumption of constant concentration of SSB.

SUPPORTING FIGURES

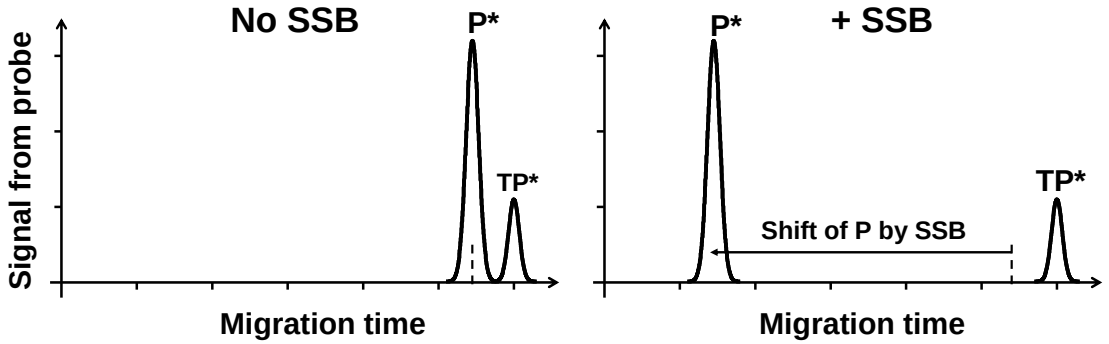

Figure S1. Conceptual illustration of the effect of SSB present in the run buffer on the capillary-electrophoresis separation of target-probe hybrid, TP*, form the excess of free probe, P*.

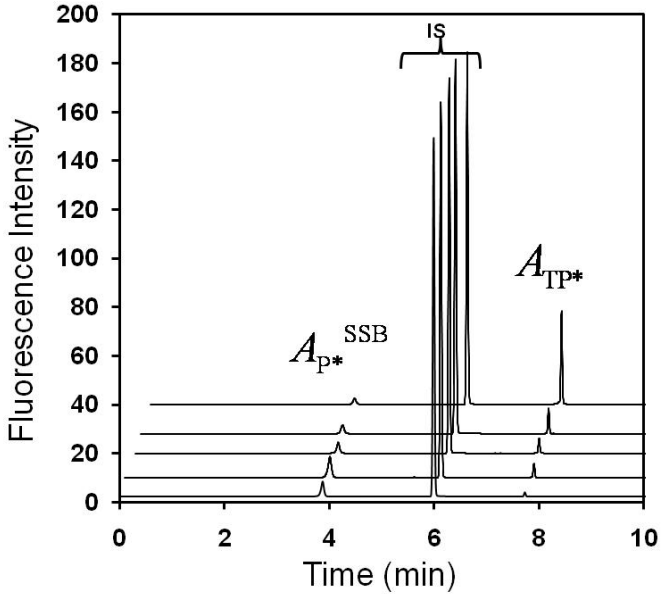

Figure S2. Electropherograms used for calculation of target, T, concentration in Fig. 7 in main text. The areas of the peaks, along with calculated quantum yields were used in determining target concentration. 100 nM DNA probe was incubated with (from bottom): 3.125, 6.25, 12.5, 25, and 50 nM target, respectively. The mixture was spiked with 100 nM fluorescein used as an internal standard (IS) and the components of the mixture were separated in the run buffer containing 50 nM SSB.