SUPPLEMENTARY MATERIAL

Tunable Blinking Kinetics of Cy5 for Precise DNA Quantification and Single-Nucleotide Difference Detection

*Hsin-Chih Yeh, Christopher M. Puleo, Yi-Ping Ho, Vasudev J. Bailey, Teck Chuan Lim, Kelvin Liu, and Tza-Huei Wang**

TABLE S1. Summary of control experiments for detection of single-nucleotide variants on *Kras* targets using the DNA three-way-junction (3WJ) method.

Exp.	w/o matching probe		w/o target		w/ longer target (80nt)[1]		w/ shorter matching probe[2]		w/ four *Kras* targets			
Reporter probe	+	+	+	+	+	+	+	+	+	+	+	+
Matching probe	–	–	–	+	+	+	–	–	+	+	+	+
Shorter matching probe	–	–	–	–	–	–	+	+	–	–	–	–
*Kras*A	–	–	–	–	–	–	–	–	+	–	–	–
*Kras*C	–	–	–	–	–	–	–	–	–	+	–	–
*Kras*G	+	–	–	–	–	–	–	–	–	–	+	–
*Kras*T	–	+	–	–	–	–	–	–	–	–	–	+
*Kras*G-80 nt	–	–	–	–	+	–	+	–	–	–	–	–
*Kras*T-80 nt	–	–	–	–	–	+	–	+	–	–	–	–
$\langle \tau_r \rangle$ (μs)	5.0 ± 0.2	5.2 ± 0.2	3.6 ± 0.1	3.6 ± 0.2	9.4 ± 0.2	6.0 ± 0.2	4.7 ± 0.1	3.7 ± 0.1	6.9 ± 0.2	6.8 ± 0.3	8.7 ± 0.5	5.9 ± 0.3
τ_d (μs)	360.0 ± 8.0	342.1 ± 9.4	235.6 ± 2.5	234.3 ± 5.8	461.9 ± 8.4	439.3 ± 7.0	427.2 ± 3.4	436.3 ± 5.2	356.5 ± 8.3	355.8 ± 16.1	360.9 ± 12.5	341.3 ± 13.5

[1] Sequence for Kras 80 nt targets is TGA AAA TGA CTG AAT ATA AAC TTG TGG TAG TTG GAG CTG **X**TG GCG TAG GCA AGA GTG CCT TGA CGA TAC AGC TAA TTC AG, where **X** is either G or T.

[2] Sequence for shorter matching probe is TGC CTA CGC CAA GAG AG.

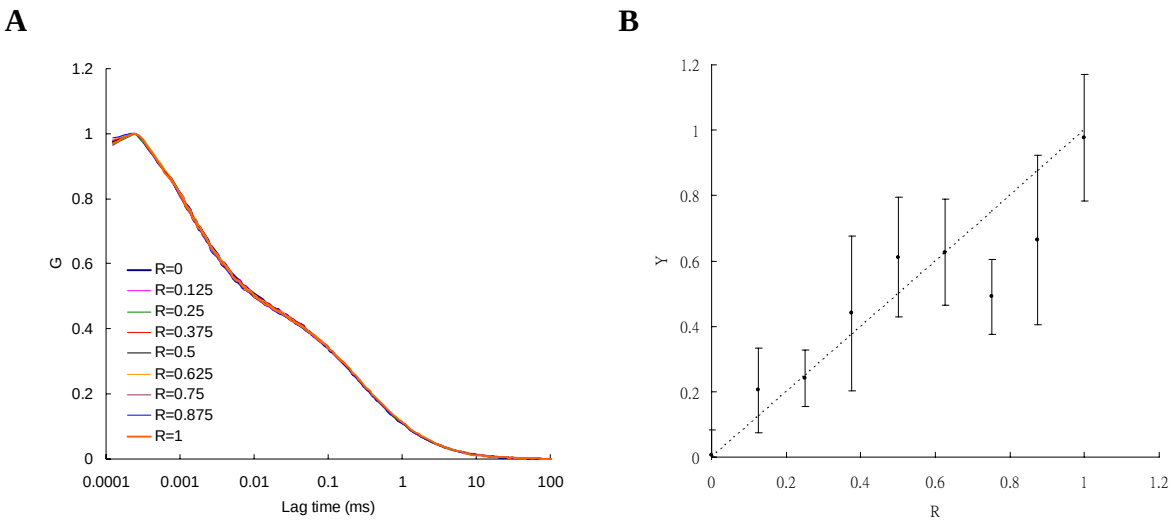

FIGURE S1. DNA quantification of a binary mixing system consisting of a different set of ssDNA having one thymine in its sequence (probe P1 in Table 1) and dsDNA (probe P1 hybridized with its complementary strand): (**A**) Autocorrelation curves of nine binary mixtures. The mean relaxation times, $\langle \tau_r \rangle$, are both 2.2 μs for dsDNA (R=1) and ssDNA (R=0). (**B**) DNA quantification results using FCS two-component analysis. All experimental conditions and analysis methods are identical to those used in experiments shown in Fig. 3.

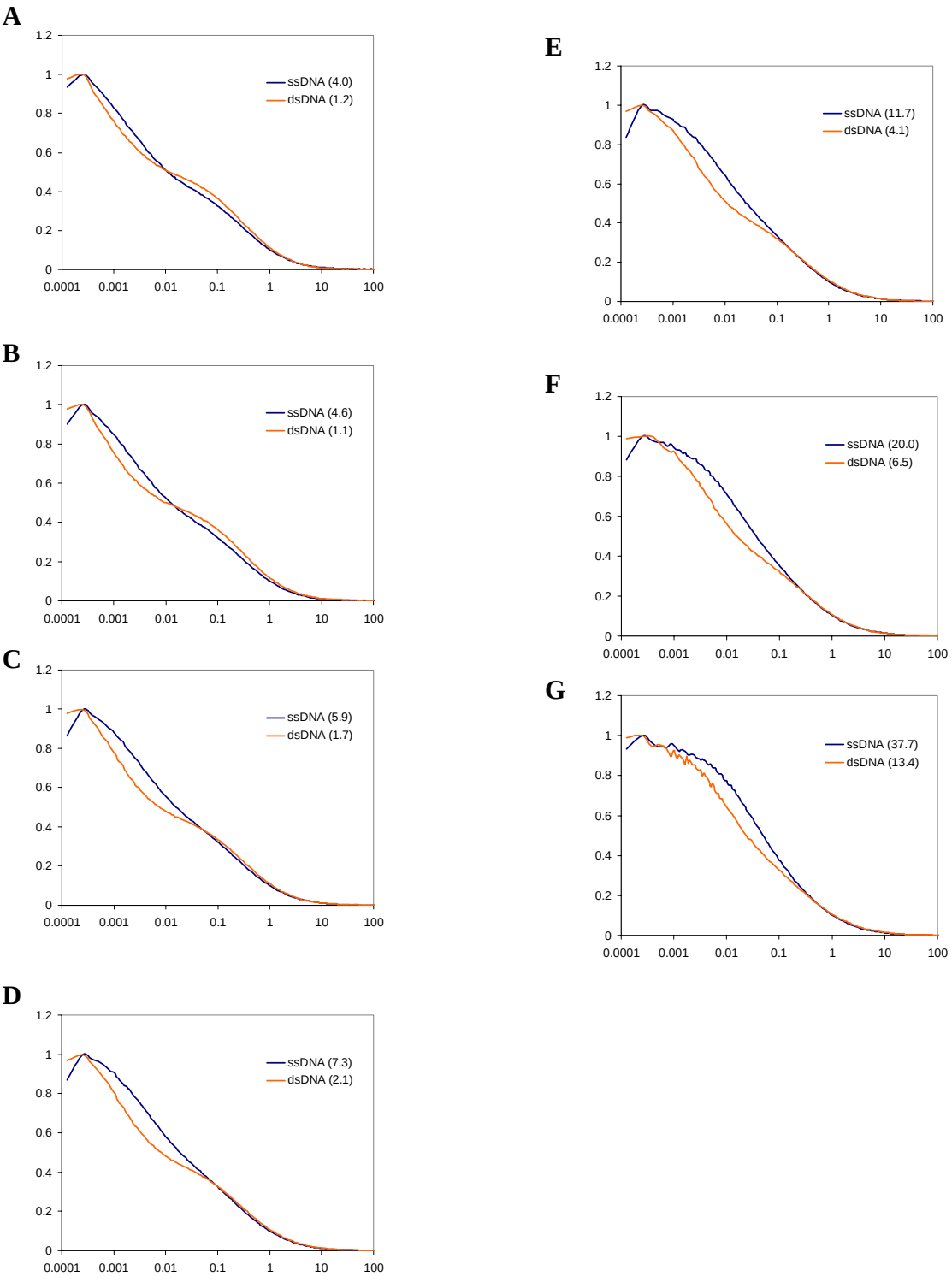

FIGURE S2. ssDNA (probe P5) and dsDNA (probe P5 hybridized with strand P5C) measured by FCS under different illumination laser powers. (**A**) Laser power before entering the objective

is adjusted to be 500 μW. (**B**) 315 μW (**C**) 160 μW. (**D**) 100 μW. (**E**) 50 μW. (**F**) 30 μW. (**G**) 15 μW. The numbers in parentheses represent the mean relaxation times in μs. The x-axis is the lag time τ in ms. The y-axis is the autocorrelation function $G(\tau)$.

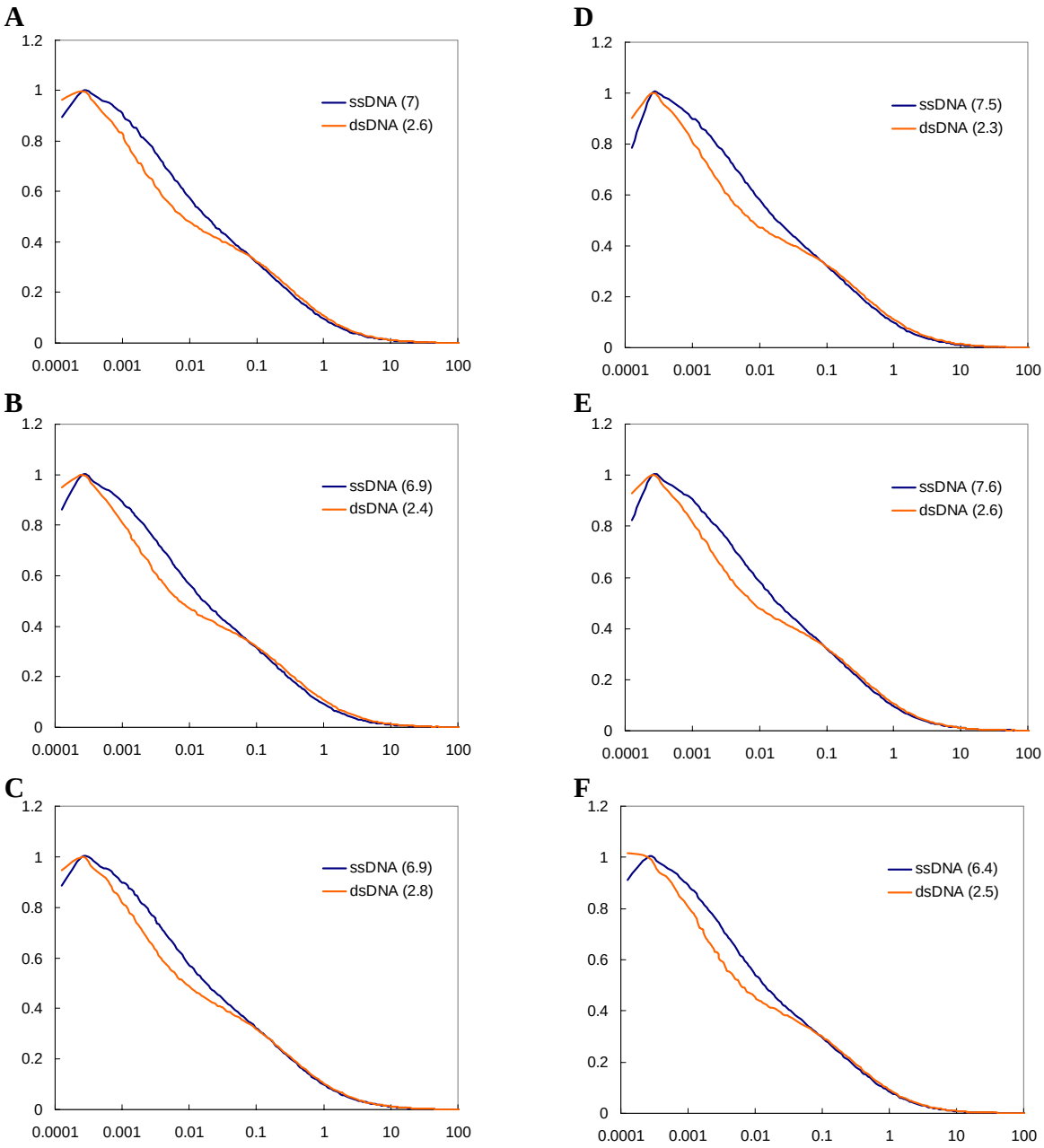

FIGURE S3. ssDNA (probe P5) and dsDNA (probe P5 hybridized with strand P5C) measured by FCS in different solutions. The dsDNA is first prepared in a 50 mM Tris-HCl (pH 8.0) buffer at 1 µM. Then it is 200-fold diluted in the following solutions. (**A**) In a buffer containing 100 mM sodium phosphate (pH 7.0) and 50 mM NaCl. (**B**) In a PCR buffer containing 40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM $(MH_4)_2SO_4$, 4 mM $MgCl_2$ and 0.2 % Triton X-100. (**C**) In a

buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$ and 0.1 % NP40 and 143 mM β-mercaptoethanol. (**D**) In a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 % NP40 and 2 mM Trolox. (**E**) In a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 % NP40 and 100 mM KI. (**F**) In water. The numbers in parentheses represent the mean relaxation times in μs. The x-axis is the lag time τ in ms. The y-axis is the autocorrelation function $G(\tau)$.

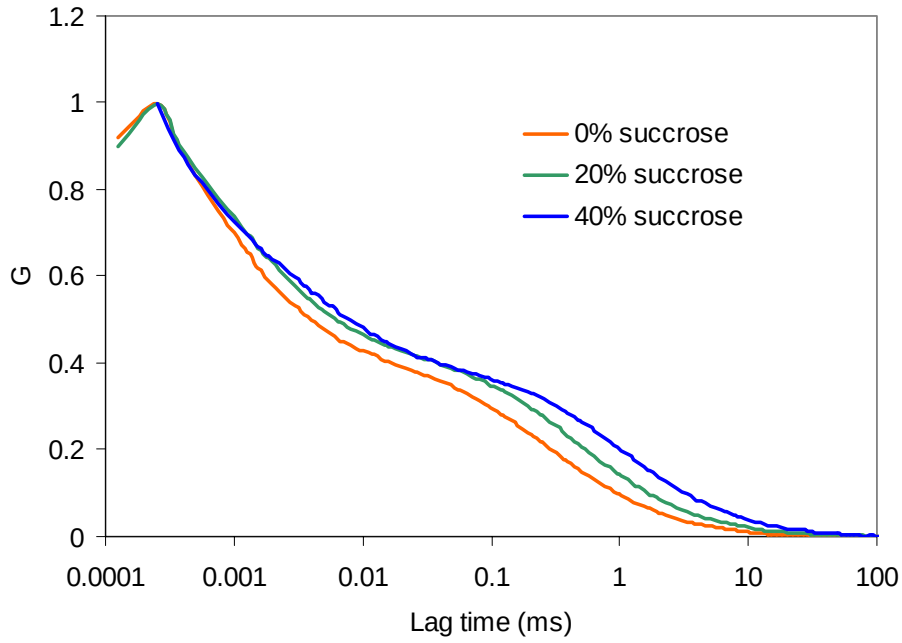

FIGURE S4. Effect of addition of sucrose on the isomerization properties of Cy5 in aqueous solution. The results are similar to the one shown in Figure 6 in Widengren et al. *J. Phys. Chem. A* 2000; 104: 6416-6428.

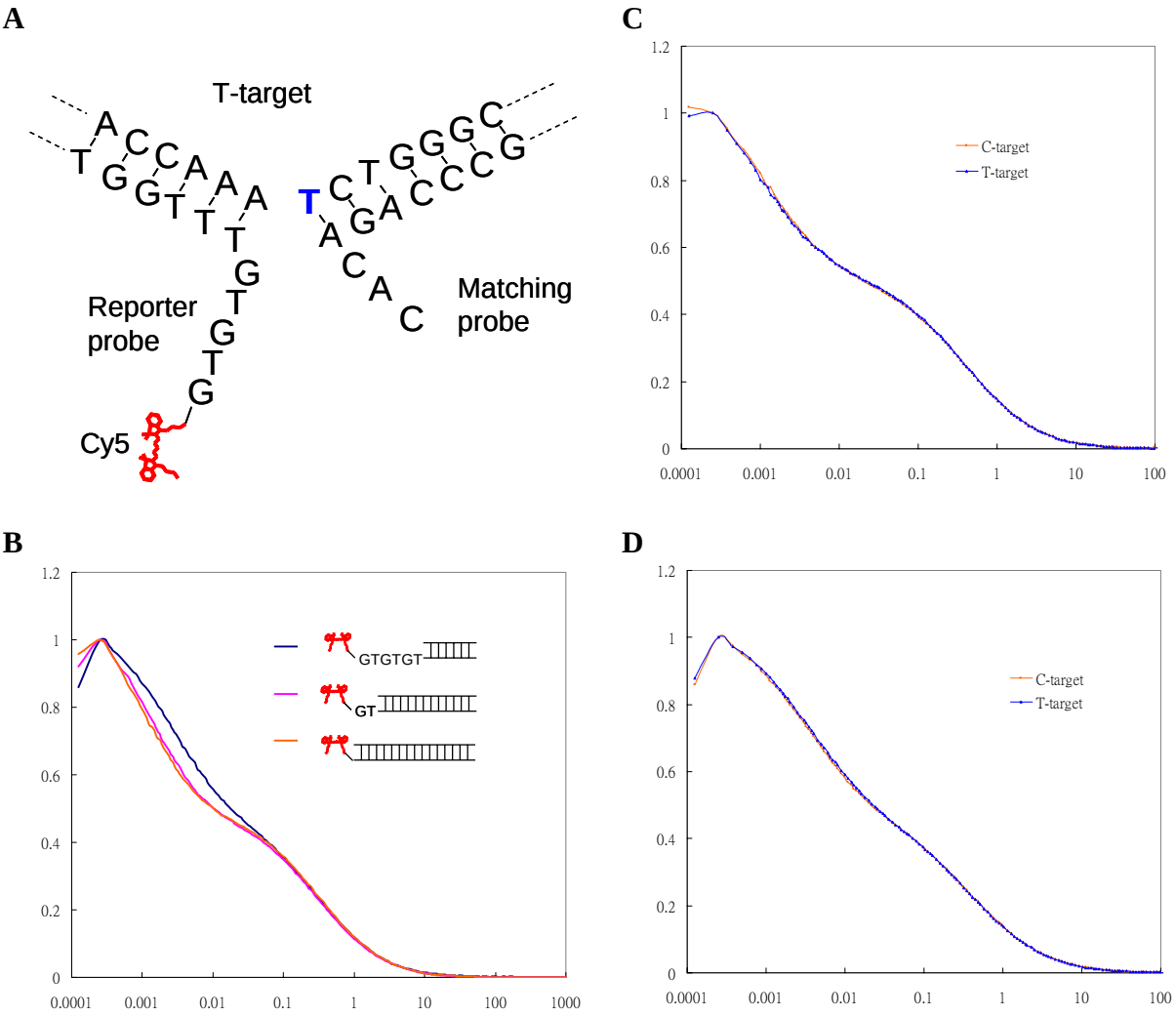

FIGURE S5. A second verification of using the 3WJ method for discrimination of single-nucleotide variants (following Fig. 6). (**A**) A schematic of DNA structure thought to form with the T-target. (**B**) Autocorrelation curves from samples containing reporter probe (22nt long) and three different-length complementary strands (16nt, 20nt, 22nt, respectively). The 16nt strand gives a GTGTGT overhang while the 20nt strand gives a GT overhang upon hybridization with the reporter probe. The 22nt strand forms blunt-end duplex with the reporter probe. It is clear that the GT overhang only slightly shifts the FBKs of Cy5. This is the reason that makes us believe the third arm does not form when T-target is used in detection. (**C**) Autocorrelation curves from samples containing reporter probe, C- or T-target, and a new matching probe (AGC CTG CCC AGA CAC AC) that is two nucleotides longer than the one used in Fig. 6. The resulting autocorrelation curves are not differentiable. (**D**) Autocorrelation curves from samples

containing reporter probe and the C- or T-target but without matching probe. The resulting autocorrelation curves are not differentiable. The x-axis is the lag time τ in ms. The y-axis is the autocorrelation function $G(\tau)$.

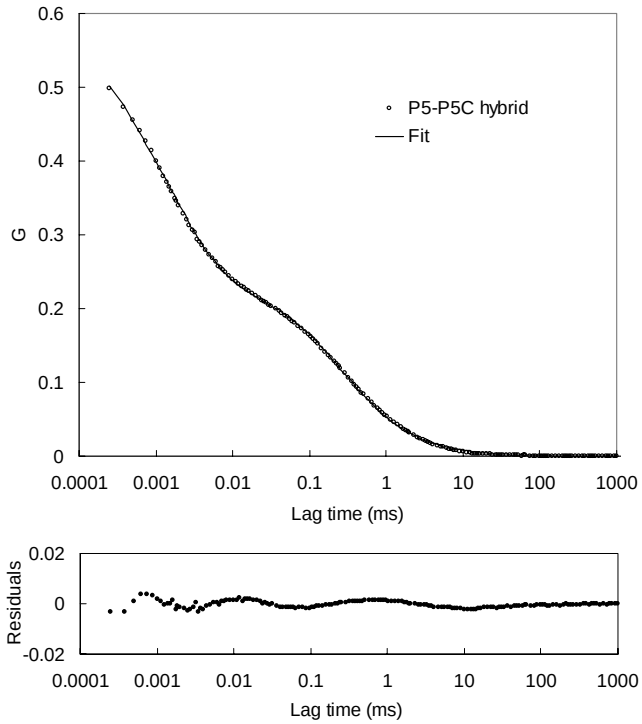

FIGURE S6. An example of the quality of fit using the one-component model.

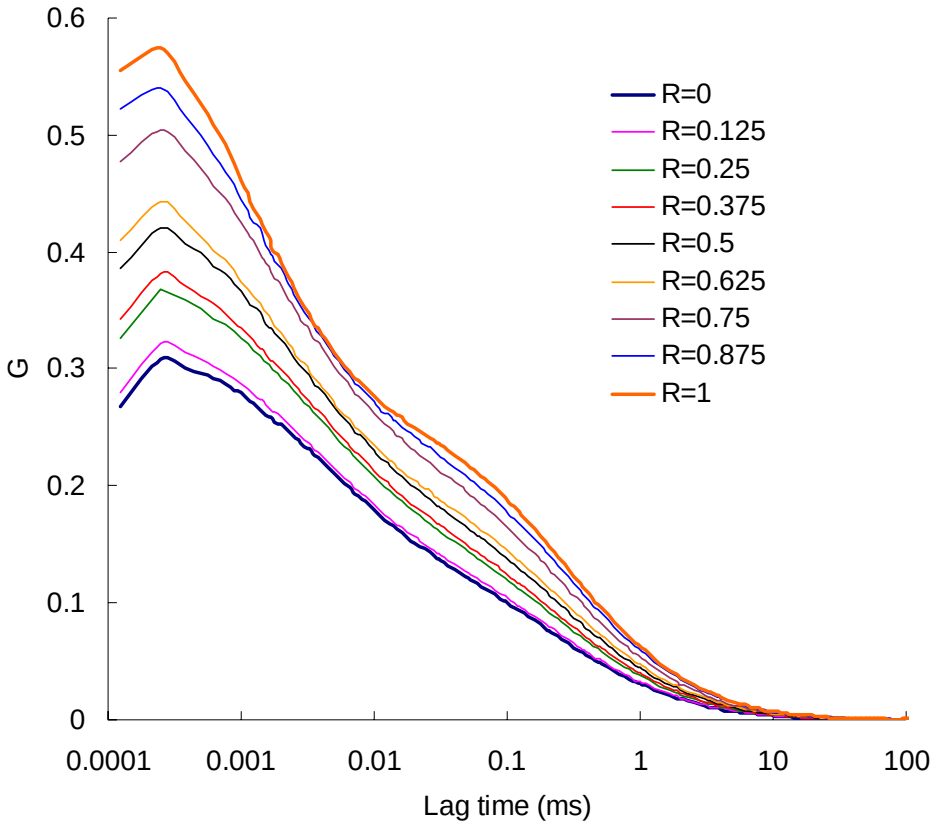

FIGURE S7. The unnormalized autocorrelation curves of nine binary (dsDNA/ssDNA) mixtures, as compared to Fig. 3, in which autocorrelation curves are normalized.