Improved RecA-assisted fluorescence assay for DNA strand exchange reaction

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Bacterial RecA protein and its archaeal and eukaryotic homologs, RadA and Rad51, respectively, are ATP-dependent filamentating DNA transferases which play a key role in homologous recombination (HR) and many other processes of DNA metabolism (1–3). In the presence of ATP, RecA forms a right-handed helical filament on single-stranded DNA (ssDNA) that is called a presynaptic filament (PF). Through multiple contact points, PF incorporates the incoming double-stranded DNA (dsDNA) to search for homology and to make a three-stranded DNA alignment, which promotes the switching of pairing and strand displacement (4). The result of this complex succession of events is the initiation of the strand exchange reaction that lies at the basis of homologous recombination in prokaryotes and eukaryotes. At the core of many practical applications of RecA known to date lies this PF-promoted DNA pairing and strand displacement, two main phases of the DNA strand exchange reaction (reviewed in Reference 5).

The most popular in vitro assays of the strand exchange are based on gel electrophoresis of the reaction products (6). However, this approach does not allow for high-throughput analysis and real-time monitoring of the reaction. The latter appears to be possible in DNA probing using fluorescence resonance energy transfer (FRET), one of sensitive reporter methods (reviewed in Reference 7). For example, fluorescein and rhodamine have been used for oligonucleotides labeling in different configurations. These donor-acceptor pairs were successfully applied to assess separately the effects of heterology on the pairing and strand displacement phases in RecA-mediated strand exchange reaction (8,9). Real-time monitoring of the reaction has been carried out due to a special configuration of labeling of single-stranded oligonucleotides (targets) and double-stranded oligonucleotides (probes) by these fluorophores. However, the potential of sensitivity through FRET has not been fully realized in these and other studies (10,11) because the ratio between homologous and heterologous signals was about 2-fold or lower.

Here, we present an improved RecA-assisted FRET-like assay that enables a high signal-to-background ratio, about 10–20 times. Two modifications of the described FRET procedures (8–11) seem to be essential. First, for molecular beacons (12) and displacing probes (13), a nonfluorescent dark quencher 4-(4′-dimethylamino phenylazo) benzonic acid (DABCYL) was used. In the above mentioned hybridization probes, both a fluorophore and a quencher were kept together in close proximity, causing the fluorescence of the fluorophore to be quenched by proximal quenching proceeding via non-FRET-based mechanisms (reviewed in References 7 and 12). Second was the use of triple repeats of the probe sequence as single-stranded targets that resulted in an increase of the effective concentration of homologous ssDNA.

The 28-mer double-stranded hybridization probe used in the study was as follows: 5′-FAM-AACTAATAAGATTTCACATTTCT-3′, 3′-DABCYL-TGTTGATTATTTCTAAAGTTGTTAAAGAG-5′, where FAM is 6-carboxyfluorescine. The 84-mer single-stranded target contained three repeats of the 5′-FAM strand: 5′-CTATAGATTTAACAATTTCTTCAAATTAAGATTTACAACATTTTCACTAAACTAATAAGTTTACATTTACAAATTTTCTCT-3′. The 101-mer single-stranded oligonucleotides used in the heterologous control were as follows: 5′-TGCAGCGTACGAGCTTACGGCCTACTACAGCTATAGACCCAGAGACGGAGAGACCTAATTGACACCGTACTACTTTGTTGTTAAAGAG-5′.

The Escherichia coli RecA protein was purified using the procedure described (14). The 125 μL reaction mixture contained 20 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 20 mM NaCl, 1 mM ATP, and its regenerating system (3 mM phosphoenolpyruvate and 30 U/mL pyruvate kinase), 10 μM ssDNA (in nucleotides), and 3 μM RecA. After a 3-min incubation at 37°C for PF formation, the strand exchange was initiated by adding 125 μL containing

![Figure 1. The real-time monitoring of DNA strand displacement promoted by (A) Escherichia coli RecA and (B) Pichia angusta Rad51 proteins.](image-url)
MgCl₂ (to a final concentration of 12 mM) and a dsDNA probe (3 μM, in nucleotides). It is noteworthy that the ratio of ssDNA to dsDNA molecules in the mixture was approximately 1:1, whereas the effective concentration of ssDNA (in nucleotides) was about three times more relative to dsDNA. The experiment and two controls are presented in Figure 1A. As expected, both controls showed very low backgrounds. As has been demonstrated earlier (8), the rate of pairing between the single-stranded target PF and double-stranded probes is an order of magnitude greater than subsequent strand displacement. This finding clarifies that the curve I in Figure 1A describes, in essence, the kinetics of strand displacement. As shown, approximately a 20-fold increase of fluorescence (relative to the heterologous control) was observed (at 2-min incubation).

In search for an optimal length of PF, the single-stranded targets of 170, 240, and 343 nucleotides (nt), but containing only one copy of the probe sequence (28 nt) within each oligonucleotide, were used. However, in all three cases the signal-to-background ratio appeared to be about 3-fold lower than that observed for the 84-mer target with a triple probe sequence (the data not shown). It means that the triple repeats are responsible for both the optimal length of PF used in the study and the efficiency of the reaction observed.

This modified FRET-like assay is especially fruitful for study on Rad51 proteins with relatively weak recombinase activities. Rad51 from a thermotolerant yeast Pichia angusta was purified as described (14). The reaction conditions were identical to those used for the E. coli RecA protein. As expected, the P. angusta Rad51 protein promoted strand displacement much more slowly than RecA but, as in the previous case, the reaction proceeded with a low background determined by the heterologous control (Figure 1B).

All data presented in Figure 1 were reproducible. The curves presented in the figure are from one typical experiment. The uncertainty in measurements did not exceed 8%.

In conclusion, two modifications of the FRET assay described here including the proximal quenching of nonfluorescent acceptor and a triple repeat of probe sequence in the target allow to increase significantly the sensitivity of the assay. The latter becomes suitable for analysis of separate steps of such a complex mechanism as DNA strand exchange managed by even weak DNA transferases. The described assay was successfully used in analysis of unusual characteristics in such DNA transferases as Rad51 from Chlamydomonas reinhardtii (V. Shalguev, O. Kaboev, I. Sizova, P. Hagemann, and V. Lanzov, unpublished results) and RecAX53, a hyperrecombinogenic E. coli and Pseudomonas aeruginosa chimeric protein (D. Baitin, D. Chervyakova, Yu. Kil, V. Lanzov, and M. Cox, unpublished results).

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COMPETING INTERESTS

The authors declare no competing interests.

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