Coupling RNA annealing and strand displacement: a FRET-based microplate reader assay for RNA chaperone activity

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Proteins with RNA chaperone activity help RNAs to obtain their native conformations, and many of them are active in the two basic reactions—RNA annealing and strand displacement. Therefore, we developed a time-saving in vitro assay that detects protein-facilitated annealing and strand displacement of fluorophore-labeled oligoribonucleotides in a microplate reader. The two reactions are followed by fluorescence resonance energy transfer (FRET) in real-time, and the effect of the proteins on the reaction constants can be quantified. The high-throughput property of the fluorescence microplate reader, the kinetic characterization, and the material-saving aspect of this assay enables a fast and convenient classification of proteins according to their RNA chaperone activity in annealing and strand displacement.

An increasing number of proteins are being found to restructure RNA either by binding to RNA and stabilizing its correct structure or by helping the RNA to resolve kinetically trapped, misfolded conformers in an action referred to as RNA chaperone activity (RCA) (1–3). Proteins with RCA are very heterogeneous and for many, their target RNAs are not well-defined. However, since these proteins associate with RNA nonspecifically, model substrates have been used to establish in vitro assays for RCA. In addition to complex systems, such as catalytic RNAs that have to be (re)folding into a native structure, the effect on simple reactions, such as RNA annealing and strand displacement can be monitored (1,4). Typically, these assays involve radioactive labeling of the substrate RNA and qualitative or quantitative analyses of gel-separated reaction products in analogy to RNA and DNA helicase assays (5,6). To better understand the mechanism and kinetics of nucleic acid hybridization, fluorescence methods have been used in steady-state approaches and in kinetic setups (7–11). However, a test system that enables a fast and comparable assessment of RNA annealing and strand displacement activities of known or putative RNA chaperones has not been established before. We therefore developed a real-time fluorescence resonance energy transfer (FRET)-based assay that takes advantage of the high-throughput capacity of a microplate reader. It couples RNA annealing with strand displacement, resulting in a convenient way to monitor the effect of proteins on these two mechanistically distinct reactions, and thereby extends a previously published assay for RNA annealing (Figure 1A) (12).

Four features contribute to the performance of this assay. (i) The use of a fluorescence microplate reader and microtiter plates allows for reaction volumes as small as 40 µL. While the microplate reader injectors are primed with the low-concentration RNA, the candidate protein can be present in the microplate wells prior to the start of the reaction and hence, only small quantities are needed. This is especially useful for RNA chaperone proteins with low-binding affinity and therefore a requirement for higher concentrations in the sample. (ii) The fact that the reader is equipped with two injectors enables the coupling of RNA annealing with a second reaction phase that either represents continuous annealing or strand displacement (Figure 1B). By including this second phase there is no need for an additional, qualitative strand displacement assay, saving thereby time and material. (iii) The evaluation of real-time data allows for a detailed kinetic analysis and determination of reaction constants. (iv) 5’-fluorophore-labeled oligoribonucleotides are commercially available, and for the Cy3™ and Cy5 pair, duplex lengths up to 35 bp result in a detectable FRET signal (13). For longer or different substrate RNA, 3’-labeling of one hybridization partner might be necessary. In contrast to most DEAD-box RNA helicases, RNA chaperones were not found to require 5’ or 3’ overhangs for strand dissociation (Figure 1 and data not shown) (14). However, the design of the RNAs can incorporate this, and additional features such as sequence specificity and structural motifs can be taken into account.

Here, two previously published fully complementary 21-nucleotide oligonucleotides were chosen (21R+, Cy5-5’-AUGUGAAAUUCACUACGAGU-3’ and 21R-, Cy3-5’-ACUGCUAGAGAUUUCACAU-3’; VBC-Biotech, Vienna, Austria) (6). Upon annealing, FRET was monitored by fluorescence emission scanning as increase in the ratio of acceptor to donor dye emission (F<sub>Cy5</sub>:F<sub>Cy3</sub>; see Supplementary Figure S1 available online at www.BioTechniques.com). RNA annealing was initiated by the injection of 20 µL 10 nM Cy5-21R+ into a well (96-well microtiter plate, black, flat bottom, half-area, medium binding; Greiner Bio-One, Kremsmuenster, Austria) containing an equal volume of 10 nM Cy3-21R- and the protein of interest. The final concentration for the phase I volume of 40 µL was 5 nM of each RNA and 1 µM protein. With Cy3 excited, both fluorescence emissions were measured once every second in a GENios Pro™ microplate reader (Tecan, Groedig, Austria). The reaction was allowed to proceed for 180 s at 37°C in a buffer containing 50 mM Tris-HCl, pH 7.5 (AppliChem, Darmstadt, Germany), 3 mM MgCl<sub>2</sub> (Merck, Vienna, Austria), and 1 mM dithiothreitol (DTT; Sigma-Aldrich, Vienna, Austria). Then, 5 µL 400-nM nonlabeled competitor RNA (21R-) were injected to yield a 10-fold molar excess over the labeled strands; the mixture was shaken vigorously for

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2 s, and readings were taken for another 180 s (Figure 1B). We performed the annealing part of this assay with the fluorescence readers POLARStar Optima (BMG Labtech, Offenburg, Germany), Varioskan Ascent® (Thermo Scientific, Langenselbold, Germany), and Victor 3 (Perkin-Elmer, Vienna, Austria), demonstrating that this assay can be carried out with a wide range of microplate readers (data not shown).

Due to the microplate reader’s intrinsic variation in both the starting points and amplitudes of the fluorescence emission readings, the evaluation of this assay focuses on the kinetics of the reactions (see Supplementary Figure S2 for further information). For better visualization and to compare the results more easily, the FRET index (ratio of $F_{\text{Cy5}}/F_{\text{Cy3}}$) was normalized to 1 at $t_{180}$ s. The time-resolved curves were least-square fitted with Prism 4.03 (GraphPad Software, San Diego, CA, USA) for phase I with the second-order reaction equation for equimolar initial reactant concentrations: $y = A(1 - 1/(k_{\text{ann,1}} t + 1))$ where $k_{\text{ann,1}}$ is the observed annealing reaction constant, and $A$ is the maximum reaction amplitude. After competitor RNA injection, phase II was assessed to be either describing continuing RNA annealing (FRET index increasing) or strand displacement (FRET index decreasing) and fitted accordingly with a single-exponential function for signal increase $y = y_0 + A(1 - \exp(-k_{\text{ann,2}} t))$ or decay $y = y_0 + A \exp(-k_{\text{SD}} t)$. Notably the observed strand displacement reaction constant $k_{\text{SD}}$ also comprises residual annealing of single-stranded RNAs.

The assay was applied to detect RNA chaperone activity of Hfq, a pleiotropic effector that is known to facilitate the interaction of small regulatory RNAs with their target mRNAs, of the tyrosyl tRNA synthetase and group I intron-specific splicing factor Cyt-18, and of L9, a protein from the Escherichia coli large ribosomal subunit (15–17). These proteins were compared with E. coli StpA, which has been shown previously to accelerate annealing and to facilitate strand displacement of the two RNAs used (4,6). StpA was purified as described previously (18).

The two RNA 21mers anneal in the absence of proteins with a rate constant $k_{\text{ann,1}}$ of 0.005 s$^{-1}$, because they do not form stable intramolecular secondary structures that would have to be resolved prior annealing (phase I, Figure 1B). About 45% of the RNA is double-stranded at the end of phase I as assessed by electrophoretic mobility shift assay (EMSA; data not shown). In phase II, annealing was accelerated due to the higher concentration of one reactant (labeled and nonlabeled 21R–), and the amplitude is quenched since only approximately 9% of the newly formed double-stranded RNA give a FRET signal. StpA was found to promote annealing about 5-fold and to stimulate strand displacement in phase II. In contrast, Hfq enhances the annealing rate 7-
fold but is not competent to facilitate strand displacement. In phase II, only a residual FRET index increase is observed indicating that annealing was near completion before competitor injection. L9 is inactive in both reaction types, which is in agreement with its lack of RNA chaperone activity in a trans-splicing assay (16). Cyt-18 does not promote RNA annealing, whereas strand displacement was induced significantly. The strand displacement rate constants for Cyt-18 and StpA are comparable ($k_{SD}$ of 0.031 s⁻¹ and 0.021 s⁻¹, respectively), however the phase-II FRET index amplitudes differ (Figure 1, B and C). Binding of proteins to nucleic acids has also been demonstrated to alter fluorescence emission for Cy3 and Cy5 labels differently and to thereby affect FRET (11) (see also Supplementary Figure S3). In addition, differential binding of single- and double-stranded RNA by Cyt-18 and StpA could be responsible for the observed FRET index divergence.

In conclusion, the results summarized in Figure 1B show that the assay readily distinguishes proteins that are active in both, none, or only one of the two reaction types. Within one measurement setup, two key RNA chaperone activities can be assigned to a protein and so far, we have successfully applied this assay to search for StpA mutants that are deficient in promoting RNA annealing but not strand displacement. In addition, we could assess more than 40 proteins for their RNA chaperone activities in a short period (data not shown or deposited at our RNA Chaperone Activity web site; www.projects.mfpl.ac.at/rnachaperones). An adaptation of the nucleic acid design would make this assay suitable for a time-saving kinetic evaluation of RNA (un)folding and catalysis, as well as for the measurement of RNA/DNA helicase activity. This might be of special interest since recently, ATP-independent RNA annealing activities of some DEAD-box RNA helicases have been published (19–21). The post-reaction accessibility of the sample in the microplate well also allows for downstream analyses including EMSA assays (e.g., to derive absolute values for the duplex formation) or emission spectrum scanning (e.g., to gain absolute FRET efficiency values).

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