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Calibration-Free Quantitative Analysis of mRNA

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Here we introduce a method for accurate and sensitive quantitative analysis of mRNA, which does not require calibration with mRNA. The method uses a fluorescently labeled hybridization probe as a reference standard. It involves the following: (i) annealing mRNA to the excess of the fluorescently labeled ssDNA hybridization probe, (ii) separation of the mRNA−probe hybrid from the excess of the probe by gel-free capillary electrophoresis mediated by ssDNA-binding protein, (iii) fluorescence detection of the hybrid and the excess probe, and (iv) quantification of mRNA using a simple algebraic formula. The method also overcomes a number of other limitations of conventional methods: the entire procedure currently takes only 2 h and accurately quantifies 10^5 copies of mRNA. With further improvements to the method, the procedure can be potentially shortened to 10 min, and the limit of quantification can be decreased to as few as 100 copies of mRNA. In this work, we prove the principle of the method by quantifying mRNA of green fluorescent protein in the matrix of total cellular RNA. The developed method is quantitative, simple, fast, and highly sensitive. It requires commercially available instrumentation only. The method will be an indispensable tool for molecular and cell biology studies.

Quantitative analysis of messenger RNA (mRNA) is crucially important in studying gene expression and diagnosing a number of disease conditions.[1−3] Conventional methods of quantifying mRNA include Northern analysis, ribonuclease protection assay (RPA), reverse transcriptase PCR (RT-PCR), and cDNA-microarray hybridization analyses with optical or electrochemical readouts. Each of these methods has certain advantages and limitations in terms of accuracy, sensitivity, material and time demands, and environmental risks. Northern analysis and RPA allow for sensitive detection of mRNA; however, the procedures are characterized by a number of limitations. Both methods are time-consuming and involve radioactively labeled probes, thus imposing radioactive hazard.[4−6] Moreover, the accuracy of Northern analysis and RPA are low, thus, rendering the methods semiquantitative. RT-PCR is much faster than Northern analysis and RPA; the procedure takes ∼4 h, instead of ∼1 week for Northern analysis. RT-PCR is also considered to be the most sensitive method with the theoretical limit of detection of as low as 1 copy of mRNA. It also allows multiplexed detection of several genes simultaneously.[7] Yet its accuracy is greatly affected by operator- and reagent-induced variations, which are amplified with the increasing number of PCR cycles.[8−12] The major advantage of cDNA-microarray hybridization analyses is spatial resolution, which facilitates simultaneous analysis of a large number of mRNAs. The high throughput is achieved, however, at the expense of low sensitivity and low accuracy.[13,14] Occasionally, different detection methods are combined to enhance the sensitivity and specificity of the system, as in the case of using competitive RT-PCR and the electrochemical detection of the overexpression of Rak nuclear tyrosine kinase[15] or as in the case of combining the thermal cycler and fluorometer to allow for more sensitive detection of the PCR products in the diagnosis of central nervous system infections.[16] Importantly, all conventional methods of quantitative mRNA analyses share a common limitation: all of them require calibration with known concentrations of pure mRNA, which involves labor-intensive and time-consuming procedures. The present work aimed at developing a method that does not require calibration with mRNA while overcoming many other limitations of the conventional methods.

We recently proposed the use of DNA-binding proteins in capillary electrophoresis (CE) for highly efficient gel-free affinity analyses of proteins and hybridization analyses of oligonucleotides.[17] Here we introduce a method for accurate and sensitive quantitative analysis of mRNA, which is based on the unique property of single-stranded (ss)DNA-binding protein (SSB) to bind ssDNA and not to bind ssRNA or DNA−RNA hybrids. This new method involves the hybridization of excess of the fluorescent

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ssDNA probe to mRNA, followed by the separation of the unbound probe from the hybrid (mRNA−probe) using SSB as a separation vehicle in gel-free capillary electrophoresis. The separated components are then detected, and mRNA is quantified using a simple formula.

Uniquely, the method does not require calibration with mRNA as it uses the probe as an internal standard. In addition, the method is fast and highly sensitive. The analysis currently takes 2 h and can accurately quantify 10^5 copies of mRNA. In this work, the method is applied to the analysis of mRNA of green fluorescent protein (GFP) from total cellular RNA. Finally, we identify approaches to further improvements to the method, which can potentially shorten the procedure to 10 min and decrease its limit of quantification to as few as 100 copies of mRNA. The new method is quantitative, fast, simple, and sensitive; it will augment the rapidly expanding toolbox of instrumental analytical methods for biomedical studies.

EXPERIMENTAL SECTION

Materials. Unless specified otherwise, all chemicals were from Sigma-Aldrich (Oakville, ON, Canada). Milli-Q-quality deionized water was used to prepare all solutions. All solutions and water were additionally filtered through a 0.22-μm filter (Millipore, Nepean, ON). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). pcDNA3.1/NT-GFP vector was purchased from Invitrogen Canada (Burlington, ON, Canada).

Hybridization Probe. The hybridization probe had the following sequence: 5′-AGGGCAGATTGTGTCGACAGGTA-ATG-GTTGT-3′. The 5′-end of the probe was labeled with 6-carboxy-fluorescein. The probe was custom-synthesized by IDT (Coralville, IA).

In Vitro Transcription and the Synthesis of mRNA. The plasmid pcDNA3.1/Nt-GFP vector, which encodes GFP, was linearized with AgeI (New England Biolabs, Ipswich, MA), and was in vitro transcribed using the mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX); the resulting capped mRNA was then used for the analysis.

Isolation of Total RNA. *Escherichia coli* bacteria expressing GFP were cultured at 37 °C overnight. The cells were then harvested and homogenized in Trizol reagent (Invitrogen Canada Inc., Burlington, ON, Canada). The samples were processed according to the manufacturer's recommendations. As a negative control, we used the total RNA from *E. coli* cells that did not express GFP. The integrity of RNA was confirmed by visualizing it on an agarose gel. The amount of total RNA used in these experiments ranged from 2 μg to 2.5 ng in the sample before injection.

Hybridization Conditions. Hybridization was carried out in a MasterCycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Pure mRNA was diluted by up to 10^5 times (concentrations ranged from 20 nM to 10 pM), mixed with the hybridization probe (20 nM) in the incubation buffer (20 mM Tris-HCl, 5 mM KCl, and 1 mM $MgCl_2$), and incubated at a denaturing temperature of 72 °C for 5 min. The temperature was then lowered to 55 °C, and the probe was allowed to anneal to RNA for 1 h. The mixture was then removed from the thermocycler and cooled to room temperature. SSB (200 nM) was added, and the mixture was incubated for 10 min. RNA is known to be prone to degradation by nucleases. To minimize the extent of this degradation, we used a nuclease-free environment for handling RNA samples. Moreover, the in vitro transcribed mRNA had a 7-methylguanosine cap structure at its 5′-end that makes mRNA less susceptible for degradation.

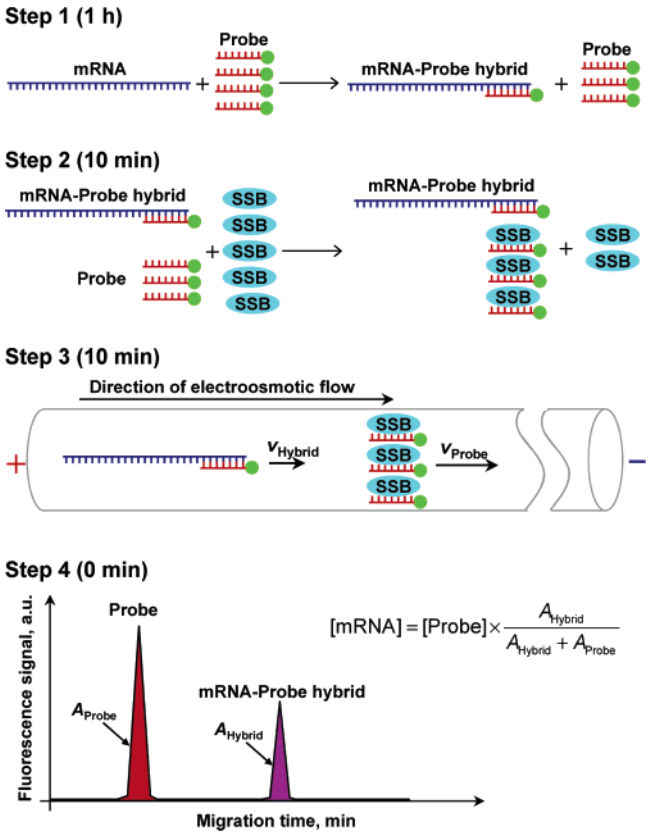

Figure 1. Schematic representation of calibration-free quantitative analysis of mRNA. Step 1 is the hybridization reaction of mRNA with the excess of the fluorescently labeled probe. Step 2 is separation the mRNA−probe hybrid from the excess of free probe by gel-free capillary electrophoresis mediated by SSB. Step 3 is the detection of the mRNA−probe hybrid and quantitation of mRNA concentration.

Capillary Electrophoresis. We used a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA) with laser-induced fluorescent detection. Fluorescence was excited with a 488-nm line of an argon ion laser. We used bare fused-silica capillaries with an outer diameter of 365 μm, an inner diameter of 75 μm, and a total length of 50 cm. The distance from the injection end of the capillary to the detector was 39 cm. The running buffer was 25 mM sodium tetraborate at pH 9.3. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, water, and the CE run buffer for 1 min each. Samples were injected by a pressure pulse of 2 psi for 10 s; the volume of the injected sample was ~0.24 μL. Electrophoresis was driven by an electric field of 0.4 kV/cm.

RESULTS AND DISCUSSION

First, we briefly explain the concept of the proposed method. The method involves four steps schematically depicted in Figure 1. Steps 3 and 4 are parts of the same procedure, which is split into two individual steps for the clarity of presentation only. In step 1, mRNA is annealed to a fluorescently labeled ssDNA hybridization probe, which is complimentary to a short part of the target mRNA. The probe is taken in large excess to mRNA to ensure that all mRNA is hybridized. The annealing reaction is

performed in a standard thermocycler and takes 1 h. In step 2, SSB is added to the reaction mixture in excess to the probe. SSB is known to bind ssDNA sequence-nonspecifically but not to bind ssRNA or double-stranded nucleic acids.[18,19] Hence, SSB binds the excess of the probe but does not bind the mRNA−probe hybrid. This reaction reaches equilibrium in 10 min.[20] In step 3, the mRNA−probe hybrid is separated from the excess of SSB-bound probe by gel-free CE with positive electrode at the injection end. To maintain quasi-equilibrium between the probe and SSB during the separation, SSB is added to the run buffer (not shown in the figure) at a concentration identical to that in the reaction mixture. SSB is a large molecule with a slightly negative charge at pH 9.3 used in this work. SSB virtually moves with the electroosmotic flow and serves as a vehicle that propels the highly negatively charged ssDNA probe. The velocity of the probe−SSB complex in electrophoresis is significantly higher than that of the highly negatively charged mRNA−probe hybrid. This difference in velocities creates conditions for efficient and fast separation of the hybrid from the excess probe in a gel-free environment. Separation takes less than 10 min. Finally, in step 4, the hybrid and the probe are detected with laser-induced fluorescence and the amount of mRNA is quantified. Detection in CE is performed on-line, inside the capillary or immediately at the exit of the capillary. Thus, detection takes no extra time. The resulting electropherogram contains two peaks. The one with the shorter migration time corresponds to the excess of the probe, and the one with the longer migration time corresponds to the mRNA−probe hybrid. The areas under the two electrophoretic peaks are proportional to the amounts of excess probe and hybrid, respectively. The concentration of mRNA, [mRNA], is then calculated based on the total concentration of the probe, [Probe], in the reaction mixture and the two areas, A_{Probe} and A_{Hybrid} using a simple formula:

$$[\text{mRNA}] = [\text{Probe}]\,\frac{A_{\text{Hybrid}}}{A_{\text{Hybrid}} + A_{\text{Probe}}}$$

Importantly, steps 3 and 4 can be performed with commercially available CE instrumentation.

Second, we experimentally prove the concept depicted in Figure 1. We applied the proposed method to the analysis of mRNA of GFP, which has a total length of 729 bases. A 31-base-long ssDNA hybridization probe labeled with fluorescein was used. The first set of experiments was performed with pure mRNA prepared by in vitro transcription of GFP-encoding DNA. As a negative control, we used a sample without mRNA of GFP. As we expected, in the absence of SSB, the hybrid could not be separated from the excess probe in gel-free electrophoresis. When SSB was included in the procedure, the hybrid and the probe were separated within 10 min (Figure 2A). Negative control showed no signal for hybrid formation. We then varied the concentration of mRNA in the sample, while keeping the concentration of the probe constant. The area of the hybrid peak (normalized by the total sum area of the two peaks) increased linearly with the concentration of mRNA (Figure 3). This result proves that the concentration of mRNA can be calculated with the above formula. We then performed a more challenging analysis of mRNA of GFP, which was within the matrix of total cellular RNA. GFP was expressed in *E. coli* bacteria, bacteria were lysed, and total RNA was extracted from the lysate. Total RNA from GFP-negative bacteria was used as a negative control. A single peak of the hybrid was observed, confirming exceptional specificity of the analysis

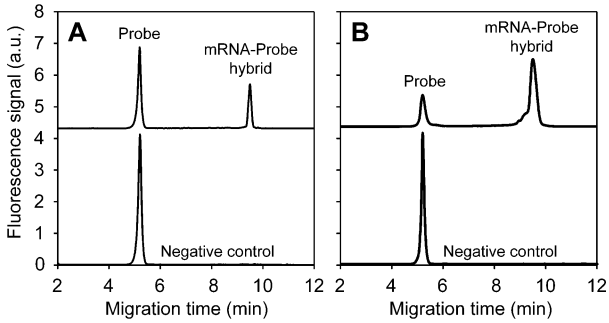

Figure 2. Gel-free separation of the mRNA−probe hybrid from the excess of the probe in the analysis of pure mRNA of GFP (A) and mRNA of GFP within the matrix of total bacterial RNA (B). Negative controls were performed in the abscence of mRNA of GFP (A) and total mRNA from GFP-negative cells (B). The analytical approach was similar to that depicted in Figure 1; experimental details are described in the Experimental Section. The traces are offset along the vertical axis for clarity of presentation.

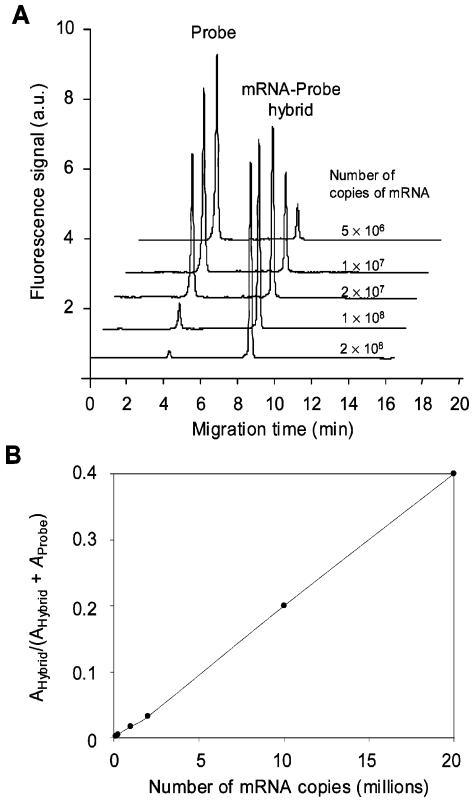

Figure 3. Quantitative features of the method for calibration-free quantitative analysis of mRNA. Panel A shows electrophoregrams for the varying number of copies of mRNA with a fixed amount of the hybridization probe. Panel B presents the normalized area of the hybrid peak as a function of the amount of mRNA.

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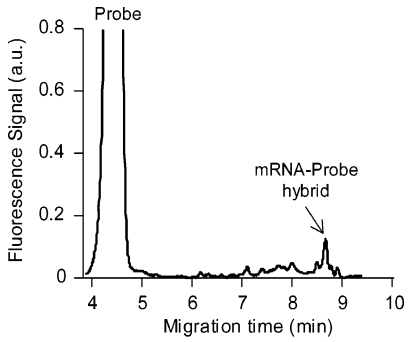

Figure 4. Limit of quantification of the method. The electropherogram shows the peak of the hybrid for the amount of mRNA, 10^5 copies, equal to the limit of quantification.

(Figure 2B). Remarkably, the quality of the analysis was virtually indistinguishable from that of pure mRNA. The only difference was a small shoulder at the left-hand side of the peak of the mRNA−probe hybrid for total RNA (Figure 2B). Total RNA was obtained from a cell lysate, which had nucleases. Even though precautions were made to eliminate their effect, minor degradation of mRNA was still inevitable. Since our method is highly sensitive, we could detect this minute degradation as a small shoulder at the base of the mRNA−probe hybrid peak. The degradation, however, does not influence quantitative features of the method: the amount of the hybrid is determined from the total areas of the peak including the shoulder.

Third, we address the issue of the limit of quantification (LOQ) of the method. LOQ is defined here as the lowest concentration of mRNA that can be measured with a signal-to-noise ratio of at least 10:1. A commercial CE instrument used in this study has LOQ of 10^4 molecules for pure fluorescein. We found that LOQ for mRNA was 10^5 copies ($\sim 1.7 \times 10^{-19}$ mol), which is only 1 order of magnitude higher than that of pure fluorescein (Figure 4). The increase was mainly due to decreased quantum yield of fluorescein when covalently bound to the probe. The volume of the injected sample was 0.24 μL of sample; thus, the concentration limit of quantitation was $\sim$0.7 pM.

The new method is perfectly quantitative with respect to mRNA due to the unique feature of the hybridization reaction. Even though it is a noncovalent reaction, the equilibrium dissociation constant of the hybrid−probe complex, K_d, is extremely low. This means that if the probe is taken in excess to mRNA, 100% of mRNA can be hybridized, thus making the recovery of the method equal to the unity. Advantageously, the time required for the "complete" hybridization can be calculated as the kinetics of hybridization can be accurately measured.

Finally, we outline approaches that can be used to make the method even faster and more sensitive. LOQ for mRNA can be further improved in a number of ways. Using a fluorescent tag with high quantum yield, which is not sensitive to conjugation, can improve LOQ by 1 order of magnitude. Some custom-made CE instruments have LOQ in the range of 10^3 molecules of a fluorescent dye, thus suggesting another order of magnitude of potential improvement. Furthermore, due to the extensive length of mRNA, as many as 20−30 fluorescently labeled probes can be hybridized on a single molecule of mRNA, thus providing an opportunity of improving LOQ by an additional order of magnitude. If all existing resources of improvement are used, LOQ can be potentially decreased to as few as 100 copies of mRNA without PCR amplification. Such sensitivity will be sufficient to quantify the expression of regulatory genes at the single-cell level.

The longest step in the analysis is the annealing of mRNA with the probe (step 1). It can be shortened from 1 h to 1 min by increasing the probe concentration from 0.02 (used in this work) to 1 μM. Increasing the concentration of the probe will increase the difference in amounts of free probe and mRNA−probe hybrid. This will require a detector with a greater dynamic range than that of the detector used in this work (such a detector is not commercially available at this time). Increasing the concentration of the probe can potentially increase nonspecific binding of the probe to other RNA. Nonspecific binding can be minimized by increasing the temperature: nonspecific hybridization for short probes has typically a melting temperature of no higher than 30 °C. To ensure the complete binding of ssDNA, the concentration of SSB in the equilibrium mixture and in the run buffer has to be increased accordingly. The procedure can be further shortened and simplified if CE is performed on a microchip. In such a case, separation (step 3) can be completed within a few seconds.[21,22] The addition of SSB to the run buffer (step 2) becomes unnecessary because the SSB−probe complex does not dissociate during this short time.[23] If these potential improvements are implemented, the entire analysis can be as short as 10 min. In addition, the method can be multiplexed for simultaneous quantification of multiple mRNAs if hybridization probes for different mRNAs are labeled with different fluorophores.

To conclude, we introduce an accurate and sensitive method for quantitative analysis of mRNA. The method uses the general concept of hybridization with relaxation of mRNA at an elevated temperature. This temperature regime ensures that any mRNA relaxes its secondary structure and makes it available for hybridization to the probe. This makes the method universally applicable to any mRNA. In contrast to conventional approaches, the new method does not require positive control with pure mRNA; the method uses the fluorescently labeled probe itself as a reference. In addition, the method is faster and simpler than the conventional methods. The limit of quantification of the method is much lower than those of Northern analysis, RPA, and cDNA-microarray hybridization analyses and comparable to that of RT-PCR. Due to its remarkable features, the method will become an indispensable analytical tool in biomedical studies.

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