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Determination of RNA degradation by capillary electrophoresis with cyan light-emitted diode-induced fluorescence

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ABSTRACT

RNA integrity plays an important role in RNA studies because poor RNA quality may have a great impact on downstream methodologies. This study proposes a cost-effective, rapid, and sensitive method for determining RNA integrity based on capillary electrophoresis that utilizes a cyan light-emitted diode-induced fluorescence as a separation tool. The capillary was initially coated with 0.1% Poly(vinylpyrrolidone) (M_{ave} 1,300,000 Da) to reduce electroosmotic flow and avoid RNA adsorption. When the capillary was filled with 0.4% poly(ethylene) oxide (M_{ave} 4,000,000) and a nucleic acid-specific fluorescent dye, SYTO 9, the baseline separation of the 18S and 28S ribosomal RNAs (rRNAs) in total RNA was accomplished within 15 min. The lowest detectable concentration for the 18S and 28S rRNAs was estimated to be 50 $\mu\text{g}/\mu\text{L}$. Some peaks longer than the 28S rRNA that migrated slowly were observed as long as the initial total RNA concentration was optimized. The temperature-induced degradation of the large RNA fragments (longer than the 28S rRNA) was faster than that of 18S rRNA and 28S rRNA. These large RNA fragments may serve as a promising marker for testing RNA integrity compared to the traditional method.

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1. Introduction

In recent years, RNA biology has received significant attention in biomedical research for RNA splicing, transcriptomics studies and small RNA regulation. Clean samples as well as RNA extraction and storage are required for this research. Unfortunately, long RNAs may degrade to short RNA fragments as a result of enzymatic digestion from sample source, temperature-induced degradation or contamination from an inappropriate operation. These sources of RNA fragmentation may degrade the experimental quality in expression microarrays or reverse transcription polymerase chain reactions. For instance, Opitz et al. demonstrated that the appropriate handling of patients samples is a prerequisite for establishing RNA quality criteria so that the samples can be used in downstream analyses [1]. Even in the case of small RNA analysis, Ibberson et al. showed that a loss of RNA quality causes poor reproducibility of microRNA expression profiles in RNA-based assays [2]. According to the literature, an RNA sample (obtained from a tissue or extracted from a cell line) commonly requires “validation” methods to verify the total RNA integrity. Determining the RNA integrity is essential before proceeding with biological experiments.

Capillary electrophoresis with laser-induced fluorescence (CE-LIF) is an important tool in bioanalytical research [3–6]. This

separation technique provides distinct advantages of high sensitivity and a low sample/reagent requirement. CE-LIF has been applied to a variety of research fields, including chiral separation [7], metabolite identification [8–10], protein separation [11,12] DNA separation, microRNA determination [13,14] and – omic studies [15]. In addition, microchip electrophoresis has been shown to be a powerful tool for bio-analysis; several sample preparation processes can be integrated into a plastic microchip. For example, Yeung et al. demonstrated that DNA extraction, PCR amplification, and DNA fragment separation can be performed in a single microchip system [16]. That study used a capture-microCE device to remove contaminating ions, concentrate the sample injection plug, and minimize electrokinetic injection bias. Waters et al. combined cell lysis, DNA extraction, PCR amplification, and electrophoresis on a monolithic microchip device [17,18]. Schoch et al. utilized isotachopheresis technology for ultrasensitive detection of small RNAs from total human RNA [19]. There are many excellent reviews of CE-LIF analysis of biomolecules [20,21]. For total RNA analysis, Zhang et al. devised a capillary array electrophoresis system and used it to evaluate mRNA expression profiling [22]. Sumitomo et al. observed that acetic acid (1.0 M, pH 3.3) can be used to separate 18S and 28S rRNAs from total RNA extracted from NIH 3T3 cells [23]. Li et al. combined pulse field capillary electrophoresis with linear hydroxyethylcellulose to separate 6000 nucleotides (nt) [24]. The ratio of $A_{260\text{ nm}}/A_{280\text{ nm}}$ of an RNA sample is a convenient way to evaluate RNA purity; however, this method cannot provide any information about RNA integrity. Despite the ratio, the quality of RNA may already be in jeopardy from digestion by

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environmental RNase or degraded by temperature-induced degradation and yet still have same absorbance at 260 nm and 280 nm. Numerous electrophoresis-based methods have been developed to evaluate RNA quality. The Agilent bioanalyzer 2100 used a plastic microchip with up to 12-wells to evaluate RNA quality [25,26]. By applying 2100 Expert software (Agilent), RNA integrity number (RIN) from 1 to 10 is obtained from eight features in the electropherograms, namely the pre-region, marker-region, 5S-region, fast-region, 18S-region, 28S-region, inter-region, precursor-region, and post-region. The RIN value can act as a reference index for downstream RNA evaluation. Opitz et al. claimed that the RIN value should be larger than 5 for an expression microarray study [1]. Ibberson et al. demonstrated that a RIN value less than 7 influences microRNA analysis [2].

Herein, the integration of CE with cyan light-emitted diode-induced fluorescence (cLEDIF) was utilized to assess RNA integrity. A poly(vinylpyrrolidone) (PVP)-coated capillary was filled with a mixture of 0.5% poly(ethylene) oxide (PEO; M_{ave} 4,000,000) and a nucleic acid-specific fluorescent dye, SYTO 9. The separation of the 18S and 28S ribosomal RNAs (rRNA) in total RNA was successfully achieved within 15 min. CE-cLEDIF was found to be well suited to detect RNase- and temperature-induced RNA degradation.

2. Experimental

2.1. Methods and materials

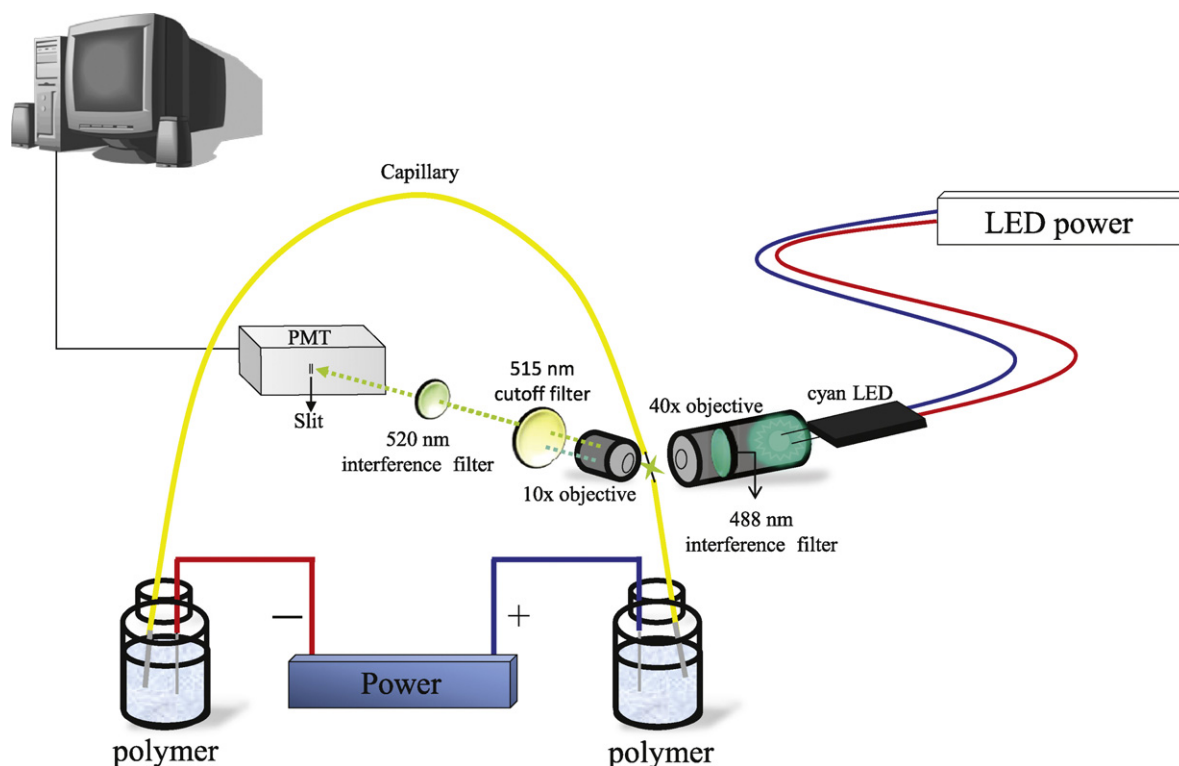
PEO (M_{ave} 4,000,000 g/mol) and PVP (M_{ave} 1,300,000 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Customized synthetic oligonucleotides (5'-Alexa Fluor 488-accacgatgtgtgtccat-3') for the LOD testing of CE-cLEDIF were purchased from Integrated DNA Technologies (San Diego, CA, USA). SYTO 9 and DNase/RNase-free water were obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.2. Cell culture and RNA extraction

The NPC cells (HK-1) were kindly provided by Prof. S.-J. Chen (Molecular Medicine Research Center, Chang Gung University). The cells were cultured in RPMI medium at 37 °C in 5% CO₂ for 3 days. The total RNA was extracted from the cell lines using a TRIzol Reagent in accordance with the manufacturer's instructions (Life Technologies, NY, USA). Before injection into the capillary, the RNA samples were heated to 70 °C for 10 min and then transferred to ice water to avoid cross-hybridization between RNA samples. DNase/RNase-free water was used to adjust the concentrations of synthetic DNA and total RNA. All DNA/RNA samples were stored at -20 °C. A prevention of repeat freeze-thaw for stock RNA sample must be taken into account due to the decreasing of the RNA integrity was observed when repeat freeze-thaw the same RNA sample more than three times.

2.3. Capillary electrophoresis with light-emitted diode-induced fluorescence

An in-house-built CE-LEDIF system was used in our laboratory with slight modification [27,28]. Briefly, a high-voltage power supply (Gamma High Voltage Research Inc., Ormond Beach, FL, USA) was used to drive electrophoresis. The entire detection system was enclosed in a black box with a high-voltage interlock. The high-voltage end of the separation system was placed in a plastic bottle for safety. A 490 nm InGaN light-emitted diode from Roithner Lasertechnik (Vienna, Austria) with a 1.2 mW total radiated power was used for excitation. The cyan LED light was projected into 40× objectives that embedded a 488 nm interference filter (Edmand, USA). The fluorescence was collected with a 10× objective (numerical aperture = 0.25), and a 520 nm interference filter combined an OG 515 cut-off filter (Edmund, Barrington, NJ, USA) was arranged after the objective to reject the scattered light before the emitted light reached the photomultiplier tube (R3896, Hamamatsu



Scheme 1. Schematic illustration of the CE-LEDIF device used for the analysis of total RNA.

Photonics, Hamamatsu, Japan). The setup of the device is represented in *Scheme 1*. The amplified currents were transferred directly through a 10 k Ω resistor to a 24-bit A/D interface at 10 Hz, controlled using chromatographic software (Clarity, DataApex, Prague, Czech Republic), and stored in a personal computer. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with a 75 μ m internal diameter were used for separation and coated with 0.5% PVP overnight, followed by 0.1% PEO for 1 h at ambient temperature. Both polymers for capillary coating were dissolved in double deionized H₂O (ddH₂O). The capillary length was 40 cm, and the effective length from the detector was 33 cm. Anodic and cathodic vials were filled with 0.4% PEO solution that was prepared in 2 \times TGA buffer (14 mM Tris, 192 mM glycine, 10 mM acetic acid, pH 7.5) [29] containing 1 \times SYTO 9 (0.5 μ M). A capillary was filled with separation medium, and then the RNA sample that diluted by ddH₂O was loaded from the inlet (cathodic) end of the capillary by electrokinetic injection, 10 kV for 10 s.

2.4. Software analysis of RNA

The reference sequences of 18S (1869 nt, NR.003286.2) and 28S (5070 nt, NR.003287.2) ribosomal RNAs were downloaded from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) website. The molecular weights of the rRNAs were calculated using Oligo 7 (Molecular Biology Insights, Inc. USA). The secondary structures of the rRNAs were predicted by the freely available software, RNAstructure (Version 5.3) [30]. The temperature settings of the RNA structural calculation were set to 25 $^{\circ}$ C and 70 $^{\circ}$ C, mimicking the handling conditions of the RNA (room temperature and partial denaturation of RNA before sample injection). The secondary structures of the ribosomal RNAs that had the lowest free energy after software calculations were chosen as representatives in *Figs. S1 and S2* (see *Supplementary Information*).

3. Results and discussion

3.1. Optical setup of cyan light-emitted diode-induced fluorescence

LEDs, which are one of the “energy-saving” light sources, have several advantages over lasers as an excitation source, such as cost-effectiveness, wide wavelength selectivity, and a larger illumination area when an LED array is assembled. However, the limitation of most LEDs is their weak radiant power and wide bandwidth, which induce a lower fluorescence and a high background relative to a laser. A cyan LED (λ_{\max} 493.2 nm) with a 1.2 mW radiant power was chosen as the excitation source for the fluorescent nucleic acid staining dye, SYTO 9. The spectrum of the LED emission (green dotted line in *Fig. 1*) displayed a broadened wavelength band (440–570 nm) that overlaps the fluorescence of SYTO 9. The use of this type of LED as an excitation source creates a strong background in electropherograms. To overcome this problem, a 488 nm interference filter was used to block LED emission above 505 nm, leading to a narrow LED emission band (475–505 nm, blue line in *Fig. 1*). When a 520 nm interference filter was employed to separate the excitation light from the fluorescence of SYTO 9, the background created by the cyan LED was efficiently minimized. A synthetic fluorescent oligonucleotide (Alexa Fluor 488; λ_{ex} : 492 nm, λ_{em} : 517 nm) was chosen to test the sensitivity of the CE-LEDIF. The linear range of CE-LEDIF could cover three orders of magnitude (100 nM to 100 pM) with a limit of detection (signal to noise ratio of 3) as low as 12.1 pM, as shown for the synthetic Alexa Fluor 488-labeled oligonucleotide in *Fig. 2*. The calibration curve of Alexa Fluor 488-labeled oligonucleotide

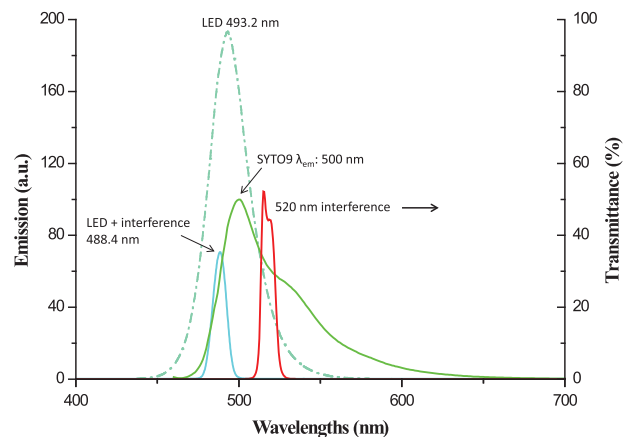


Fig. 1. The emission spectra of cyan LED, cyan LED attached to a 488.4 nm interference filter, SYTO 9 and the transmittance of interference filter attached to PMT. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

is $y = 2.3367^{12}x + 2.8897^{10}$ with $R^2 = 0.9995$. This result indicates the sensitivity of CE-cLEDIF is useful for PCR-based technologies such as DNA sequencing [31] and genetic mutation detection [32–34].

3.2. Polymer concentration affects the separation efficiency of ribosomal RNA

In traditional RNA tests, mRNA “transcripts” are of more interest to scientists than other RNA molecules; the quality and quantity of the mRNA affects its downstream biological function. However, of total RNA, only 1–5% being consisted of more than ten thousand different transcripts, and their length is approximately 2000–4000 nt. The difficulty lies in observing mRNA degradation in a total RNA sample using an electropherogram. Because PVP has been shown to be efficient at reducing EOF and DNA adsorption [35], the capillary was coated with 1% PVP prior to the separation. *Fig. 3* shows the separation of a mixture of rRNAs in the presence of 0–0.8% PEO. RNA molecules have a similar ratio of charge to mass, and in the absence of PEO, it was not possible to

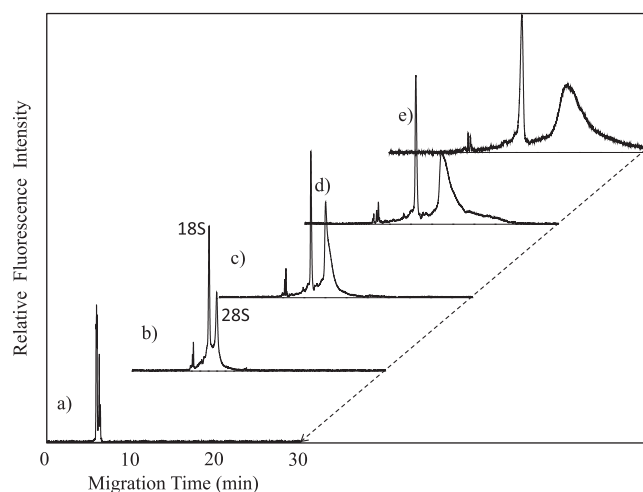


Fig. 2. Detection limit of Alexa Fluor 488 labeled oligonucleotide by cyan light-emitted diode-induced fluorescence. The sample concentrations were (a) 0.1 μ M, (b) 10 nM, (c) 1.0 nM, or (d) 0.1 nM. The experimental conditions are separation voltage: 10 kV, and separation media 0.4% PEO. The polymer solutions were prepared in 2 \times TGA buffer (pH 7.5). The DNA was electrokinetically injected into the capillary for 10 s.

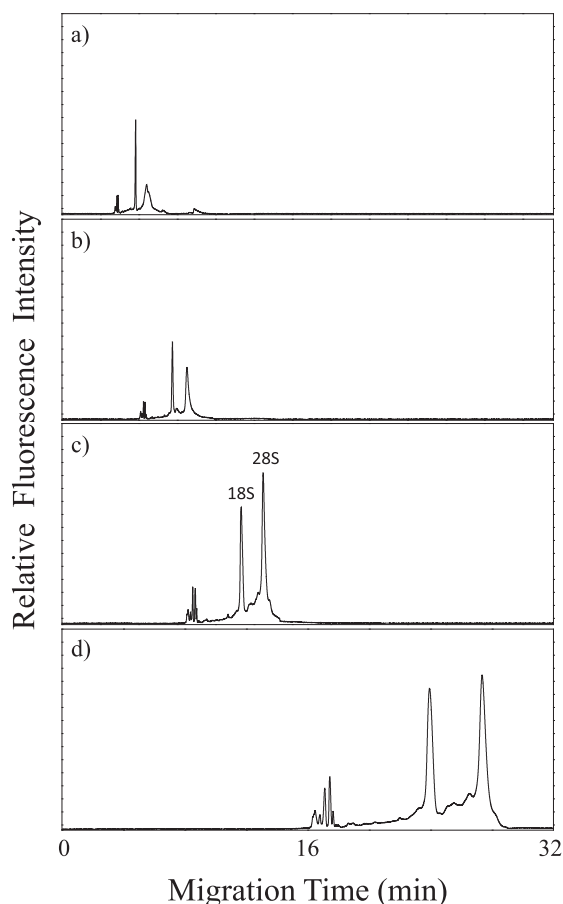


Fig. 3. Polymer concentration effect on separation efficiency. The experimental conditions are separation voltage, 10 kV, and separation media: (a) 2× TGA, (b) 0.2% PEO, (c) 0.4% PEO, (d) 0.6% PEO, (e) 0.8% PEO. All polymer solutions were prepared in 2× TGA buffer (pH 7.5). The total RNA sample (HK-1) was electrokinetically injected into the capillary for 10 s.

separate them (Fig. 3a). By varying the PEO concentration from 0.2% to 0.8%, the three major RNA components (small RNA, 18S rRNA, 28S rRNA) were found to be well separated at 0.4% PEO (Fig. 3b–e). The migratory behavior of rRNA follows Ogston theory, so the migration time of an rRNA increased with increasing length. Therefore, the migration times of 28S (~4700 nt) and 18S (~1900 nt) rRNAs are longer than those of 5.8S (~160 nt) and 5S rRNAs (~120 nt). In the testing of RNA integrity, the peaks corresponding to 5.8S and 5S rRNAs are ineffective for evaluating 18S and 28S rRNA fragmentation. Above 0.4% PEO, the resolution between 28S (~4700 nt) and 18S (~1900 nt) rRNAs was larger than 1.2. To reduce the separation time, 0.4% PEO was used in the following studies.

3.3. Separation voltage effect on rRNA electropherograms

Previous studies have shown that the structure of a large DNA molecule is susceptible to an external electric field [36,37]. We reasoned that the separation efficiency of RNA can be improved by varying the external electric field. Under high voltage (20 kV), the migration times of rRNAs were shortened to approximately 5 min, at the expense of separation efficiency (Fig. 4a). These results indicate that the rRNAs might dissociate under a high electric field (500 V/cm). In another experiment (Fig. 4b), when the separation voltage dropped to 15 kV, the peak height of the 28S rRNA increased two-fold, but the 18S and small RNAs remained consistent. This

result is in agreement with Yeung et al., who observed the breakdown of a single DNA molecule when a high electric field was applied to the capillary [38,39]. Furthermore, the 28S to 18S ratio also exhibited an increase as the electric field dropped again to 250 V/cm (Fig. 4c). These results indicate that a separation voltage smaller than 10 kV (40 cm capillary) is appropriate to separate a large RNA molecule. A comparison of the fluorescence intensities of RNA molecules between Fig. 4b and d indicates that a decrease in the electric field induces the enhancement of peak intensities of RNA molecules. The intensities of the small RNAs region (tRNA, 5S rRNA, 5.8S rRNA) shared a similar trend to that of the larger RNA molecules; therefore, this phenomenon is believed to contribute to the separation voltage that affects the association between the RNA molecule and SYTO 9. These results agree with previous work that claimed that the binding between fluorescent dyes and DNA molecules dissociates under high electric fields [40,41]. To reduce the separation time, our data suggest that 0.4 M PEO be used under a 250 V/cm electric field; these conditions are good for total RNA integrity testing.

3.4. Electropherograms affected by total RNA concentration

To test the effect of the RNA concentration on separation performance, varying concentrations of human total RNA were separated under 250 V/cm in the presence of 0.4% PEO. When 500 ng/ μ L total RNA was detected by the present method, the observation of a single peak reflected the unsuccessful separation of the 18S and 28S rRNAs. These results were not surprising; there was only a limited number SYTO 9 molecules that could interact with the RNA molecules within that particular separation time. For instance, the capillary contained 0.35 ng of SYTO 9, which was calculated from the concentration of the dye dissolved in the polymer solution (0.5 μ M) and the length of capillary from the inlet to the detection window (33 cm, 1.8 μ L). SYTO 9 is a cyanine-type molecule and carries a positive charge when dissolved in a particular buffer [42]. Therefore, SYTO 9 may migrate from the anode, against the RNA molecules, through the capillary. To evaluate the migration rate of SYTO 9 under a particular electrophoretic condition, the amount of SYTO 9 that migrates into the capillary must be calculated until just the 28S rRNA migrates to the detection window (<14 min); that corresponds to approximately 0.35 ng of SYTO 9. Based on the migration rates of the small RNAs corresponding to the 18S and 28S rRNAs within the polymer solution, SYTO 9 (0.7 ng) may be limiting and able to incorporate only a partial amount of small RNAs (tRNA, 5S and 5.8S) when the RNA migrates to the anode end of capillary. The poor resolution observed in Fig. 5a (500 ng/ μ L) indicates that 1) there is cross-hybridization between different RNA molecules at high concentrations of RNAs and that 2) SYTO 9 exchanges between the same RNAs at relatively low concentrations. This phenomenon was illustrated in Fig. 5b and c. At a ten-fold dilution, the total RNA is 50 ng/ μ L (Fig. 5b), and the small RNA regions (tRNA, 5S and 5.8S rRNA) are clearly indicated in the electropherogram, while the 18S and 28S rRNAs are not well resolved. The unincorporated RNA molecules will migrate faster than the dye-incorporated RNA molecules (they are neutralized by positively charged SYTO 9). Therefore, the small RNA region in Fig. 5b and c had shortened migration times compared to those in Fig. 5a as a result a lower total RNA concentration, reducing the cross-hybridization between large RNAs and small RNAs. Until the total RNA concentration decreased to a comparable level with SYTO 9 (3.12 ng versus 0.7 ng), three of the major components of total RNA including short RNAs, 18S rRNA and 28S rRNA were displayed in the electropherograms (Fig. 5c–e) and their migration times remained constant. Under this circumstance, the detectable concentration of 18S and 28S rRNAs was 50 pg/ μ L (Fig. 5e). These results achieved the same analysis range of total RNA (RNA 6000

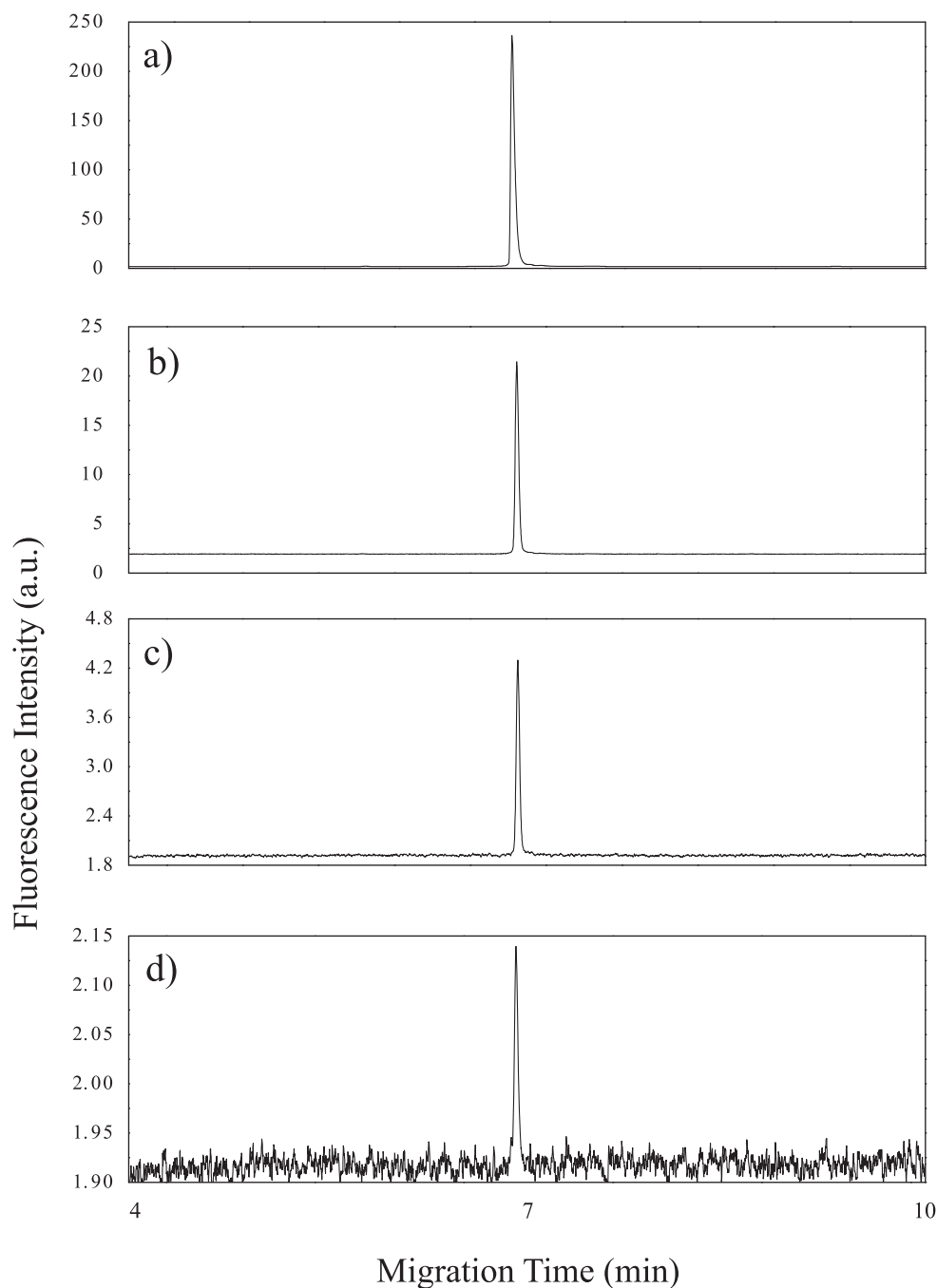


Fig. 4. Electric field effect on separation efficiency. The separation voltages are (a) 20 kV, (b) 15 kV, (c) 10 kV, or (d) 5 kV, and the other experimental conditions were the same as those in Fig. 2.

Pico kit: 50 pg–5 ng) obtained by chip electrophoresis with laser-induced fluorescence (Bioanalyzer 2100, Agilent) [43]. This finding indicates that the excellent sensitivity of CE-cLEDIF is sufficient for biomedical applications to minimize RNA sample consumption.

3.5. Temperature-induced fragmentation of RNA

Mature messenger RNAs are single-stranded RNA molecules with lengths between 2000 nt and 4000 nt. There were ten thousand large molecules pooled in the container that must have had self-annealing secondary structures that could affect the probe or primer hybridization. As shown in Fig. 6a, the 18S and 28S

rRNAs displayed a narrowed peak width compared to that shown in Fig. 6b. The sharp peak at the 18 rRNA shown in Fig. 6a might be due to the lower free energy (more compact structure) within the molecules. For example, the free energies of 18S rRNA at 25 °C and 70 °C are –936.5 and –251.9 kcal/mol, respectively. After RNA samples were heated to 70 °C for 10 min and immediately cooled in ice water, fewer hydrogen bonds formed in the 18S rRNA molecule than expected. Therefore, an opened single-strand portion may increase the molecular distribution within the capillary due to the random coil conformation during electrophoresis. Therefore, the peak profile of the 28S rRNA, as shown in Fig. 6a, was similar to that of the 18S rRNA that broadened. The 28S rRNA region resolved into three peaks (Fig. 6b), and the migration time

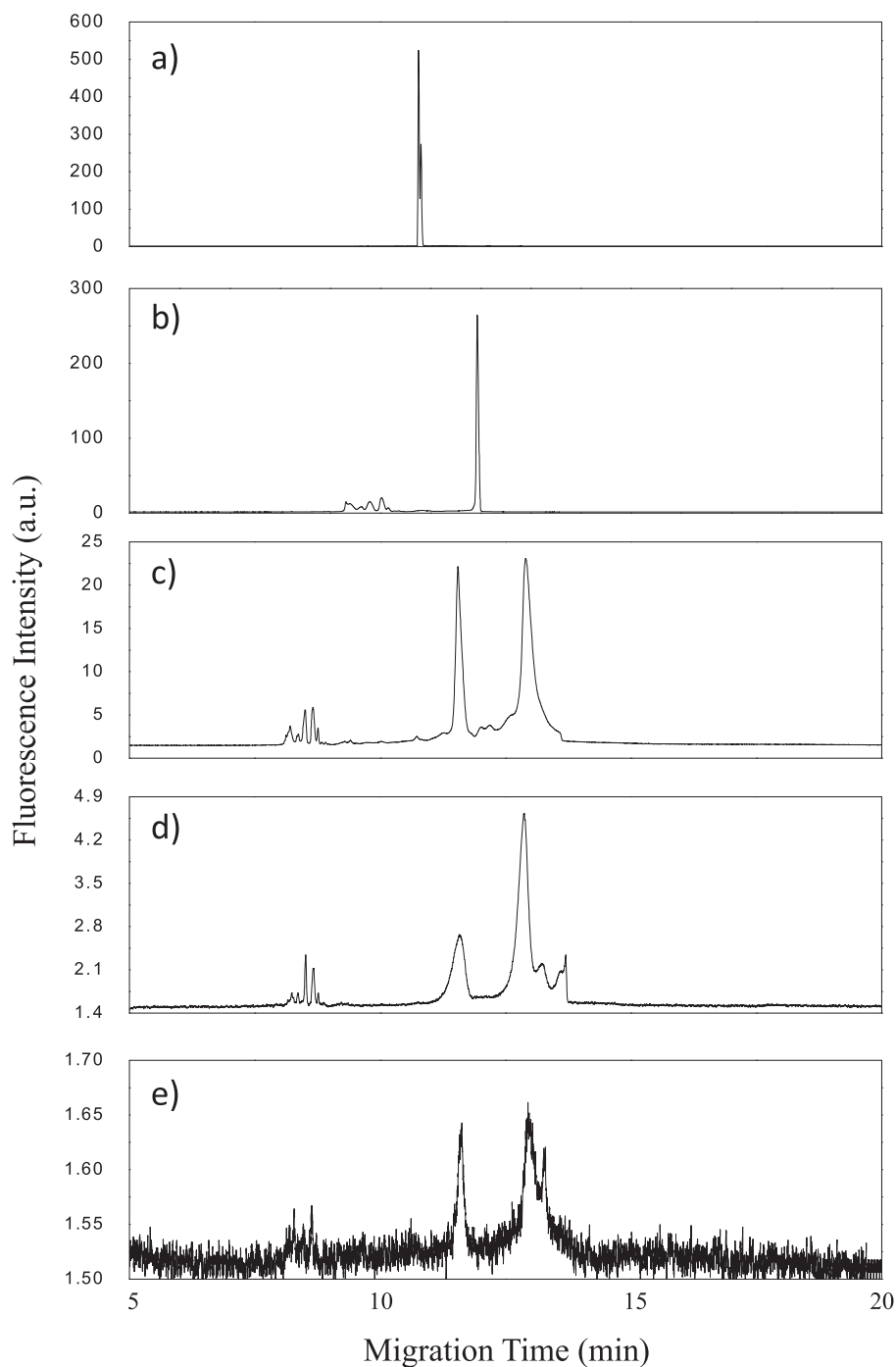


Fig. 5. Detectable limit of total RNA by cLEDIF. The concentration of each sample was (a) 500 ng/ μL , (b) 50 ng/ μL , (c) 5 ng/ μL , (d) 500 pg/ μL , or (e) 50 pg/ μL . The other experimental conditions were the same as those in Fig. 2.

remained same as that shown in Fig. 6a. As shown in Fig. S2, the 28S rRNA may have partially opened because of heating the sample at 70 °C from room temperature (Fig. S2a). The peaks with migration times slightly slower than 28S rRNA were a result of the unwound secondary structure that migrated more slowly than others, as observed in Fig. 6c and d. This result indicates that the 28S rRNAs may hybridize to each other or with other RNA molecules in a low-temperature environment, which is why many RNA protocols require a short-term heating step to unwind the self-hybridized or cross-hybridized RNA molecules [44]. The electrophoretic shifts (migration time large than 28S rRNA in Fig. 6b) of Fig. 6d shows that the temperature denatured the RNA (dsRNA \rightarrow ssRNA). The heating

process also leads to RNA degradation, similar to that observed by RNase [1]. To demonstrate the application of CE-cLEDIF for RNA fragmentation, a time-lapse study (10 min – 24 h) of RNA heating was used in this study. When the heating time rose to 1 h, as shown in Fig. 6c, the peaks of the 18S and 28S rRNAs migrated faster than those in Fig. 6b. The small RNAs region also decreased the migration time, as observed in Fig. 6c–e, after an 8 h incubation. Furthermore, the increased intensity of the small RNA region between Fig. 6c and e also support the conclusion that larger RNA molecules were fragmented. Therefore, these data indicate that the temperature-induced RNA degradation occurred and could be monitored by CE-cLEDIF.

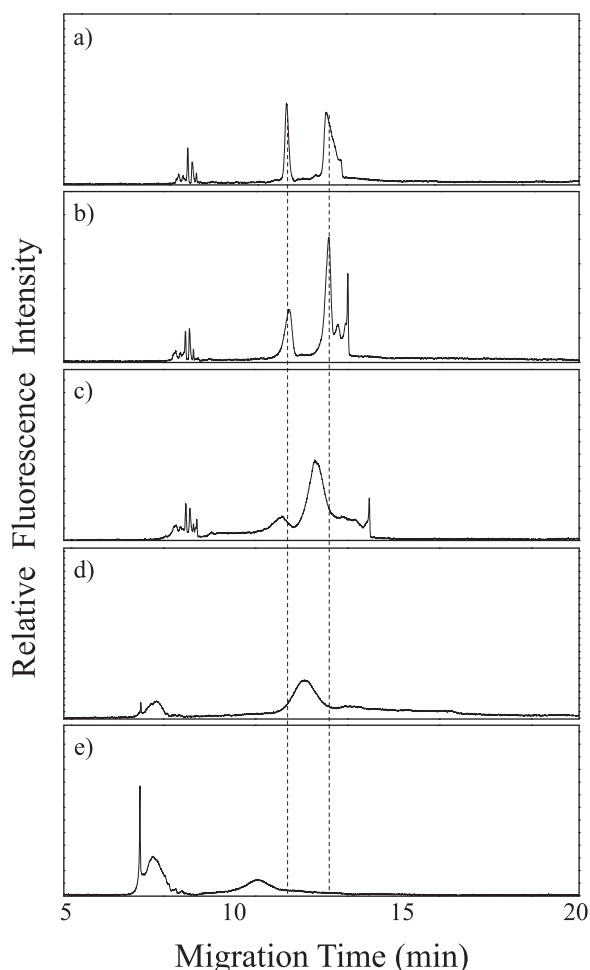


Fig. 6. Temperature-induced degradation effect on RNA integrity. The temperatures were (a) no preheating, (b) heating for 10 min at 70 °C, (c) heating for 60 min at 70 °C, (d) heating for 8 h at 70 °C, or (e) heating for 24 h at 70 °C. The other experimental conditions were the same as those in Fig. 5d.

4. Conclusions

We demonstrated the use of CE coupled to cLEDIF to measure RNA degradation. A mixture of 18S and 28S rRNAs was fully separated in the presence of 0.4% PEO. The present method provides high sensitivity (lowest detectable concentration of 50 pg/ μ L) and acceptable linear range (50 pg–5 ng). These features are comparable to those of the Agilent bioanalyzer 2100. In addition to SYTO 9, the integration of cyan LED and 488-nm interference filter should be useful to excite other organic dyes for nucleic acids labeling, such as SYBR green ($\lambda_{\text{ex}} = 497$ nm), SYBR gold ($\lambda_{\text{ex}} = 495$ nm), and YOYO-1 dye ($\lambda_{\text{ex}} = 491$ nm). The selection of an appropriate fluorophore is helpful to improve detection sensitivity. The use of an LED as an excitation source has many advantages: low cost, small size, long life time, and ease of installment into instruments with an array format [22,45]. We believe that CE-cLEDIF has great potential for clinical healthcare because it can quickly and accurately determine RNA quality.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2012.03.070>.

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