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Multiplexed microRNA detection by capillary electrophoresis with laser-induced fluorescence

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ABSTRACT

In this study, we developed a novel assay that simultaneously detects multiple miRNAs (microRNAs) within a single capillary by combining a tandem adenosine-tailed DNA bridge-assisted splinted ligation with denaturing capillary gel electrophoresis with laser-induced fluorescence. This proposed method not only represents a significant improvement in resolution but also allows for the detection of multiple miRNAs within a single capillary based on the length differences of specified target bridge DNA. The assay's linear range covers three orders of magnitude (1.0 nM to 1.0 pM) with a limit of detection (S/N = 3) as low as 190 fM (2.5 zmol). Five miRNAs of Epstein-Barr virus (EBV) were also detected in EBV-infected nasopharyngeal carcinoma cells, while they did not appear in non-virus infected cells. Moreover, the electropherogram indicated that the screening of isomiRs (isomer of miRNA) of BART2 by CE-LIF is feasible by our proposed method. The developed electrophoresis-based method for miRNA detection is fast, amplification-free, multiplexed and cost-effective, making it potentially applicable to large-scale screening of isomiRs.

1. Introduction

In recent years, researchers have discovered that microRNAs (miRNAs) are related to cancer formation, neurodegeneration, and virus-induced diseases and have thus become an important biomarker for early diagnosis [1] and for the analysis of biological processes. In the case of carcinoma, the levels of miRNAs that may be over- or underexpressed in specific tissues depend on the characteristics of the relevant oncogene or tumor suppressor gene [2]. The overall expression levels of miRNAs also play an important role in systems biology studies [3]. Therefore, a high-throughput and cost-effective assay is required for fast screening of comprehensive populations and conditional cases [4].

Until now, microarray-based platforms have been one of the most powerful tools for miRNA profiling [5,6]. These methods provided the high-throughput advantage respect to high level specificity. Additionally, numerous miRNA assays based on solid-phase hybridization have been reported, such as electrochemical and bioluminescent techniques [7,8]. More recently, Qavi and Bailey reported a multiplexed and label-free miRNA assay based on microring resonators [9]. This technology exhibited a good linear range over three orders of magnitude and was able to detect four miRNAs in U87 cells. In addition, Li's group reported a direct quantitative method based on evanescent wave-induced fluorescence. The method allowed detection limit of miRNA down to single molecule level and minimized the sample pretreatment procedure. All of these methods provide excellent sensitivity and toward miRNA detection without PCR amplification [10].

In the past decade, CE-LIF has emerged as a powerful tool for protein separation [11–13], DNA sequencing [14–16] and genotyping [17–19]; it has also been extensively used in – omics level studies [20,21]. For example, Yeung's group reported an excellent method for high-throughput RNA expression analysis based on CE-LIF in the past few years [22]. CE-LIF provided distinct advantages, such as good separation efficiency, high sensitivity, and smaller sample requirements; it can also be automated to facilitate high-throughput screening. In addition to mRNA, Zhong's group combined rolling circle amplification with CE-LIF for small RNA detection and the detection limit of this approach was down to 35 amol [23]. Furthermore, Santiago et al. pioneered the detection of small RNA using isotachophoresis preconcentration integrated microfluidic chip [24,25]. Recently, our group showed miRNA of Epstein-Barr virus (EBV) could be analyzed from nasopharyngeal carcinoma (NPC) cells by CE-LIF [26]. In this study, fluorescent dye-labeled DNA probe was directly hybridized to EBV miRNA followed by electrophoretic separation in high-viscosity linear polymer solution. Unfortunately, this method can only detect one

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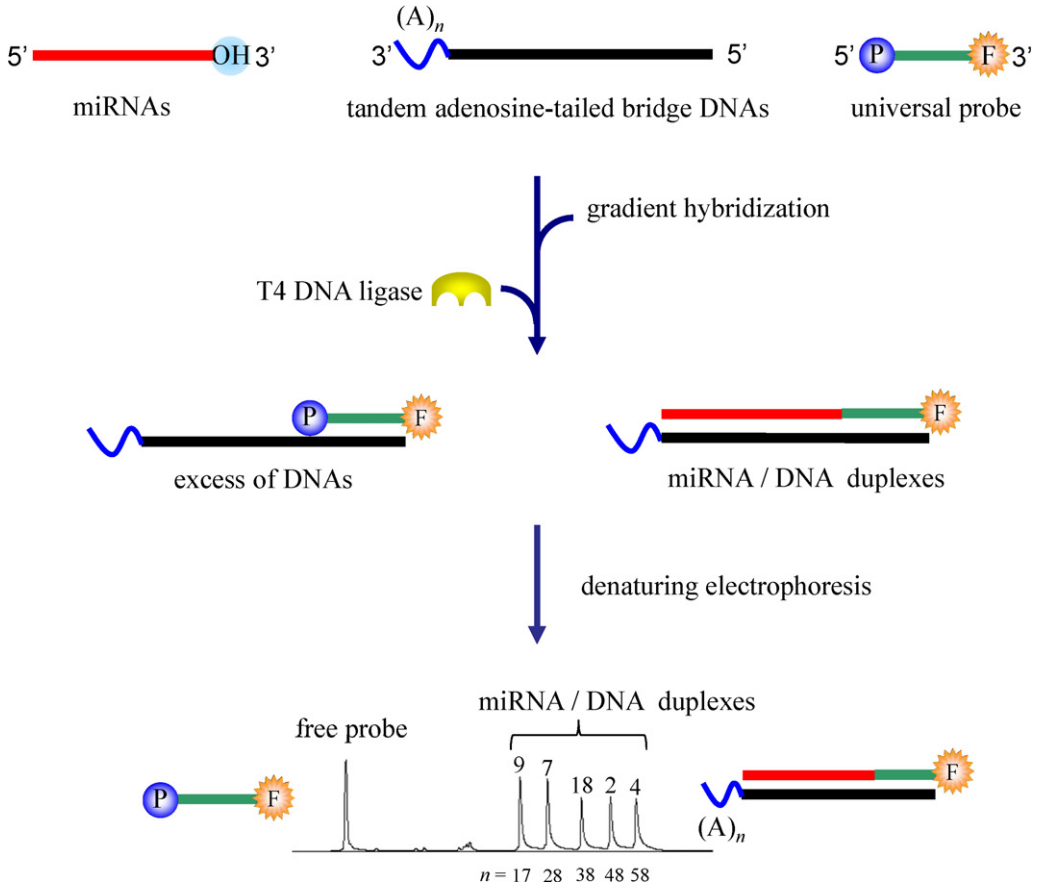
Table 1
List of oligonucleotides used in this study.[a]

Name	Length	Sequence (5′–3′)	T_m
Fluorescent probe	10	Phos-TCGGTCAGCA-Alexa Fluor 532	35.7
BART7-RNA	22	CAUCAUAGUCCAGUGUCCAGGG	67.8
BART7	22	CATCATAGTCCAGTGTCCAGGG	59.0
BART9	23	TAACACTTCATGGGTCCCGTAGT	60.2
BART18	22	TCAAGTTCGCACTTCCTATACA	56.2
BART2	22	TATTTTCTGCATTCGCCCTTGC	59.0
BART2 − 2 nt	20	TTTTCTGCATTCGCCCTTGC	59.1
BART2 + 2 nt	24	ACTATTTTCTGCATTCGCCCTTGC	61.2
BART2 + 5 nt	27	CAGACTATTTTCTGCATTCGCCCTTGC	63.4
BART4	22	GACCTGATGCTGCTGGTGTGCT	64.5
BART9-Bridge	50	*TGCTGACCGA***ACTACGGGACCCATGAAGTGTTA**(17A)	
BART7-Bridge	60	*TGCTGACCGA***CCCTGGACACTGGACTATGATG**(28A)	
BART18-Bridge	70	(19A)*TGCTGACCGA***TGTATAGGAAGTGCGAACTTGA**(19A)	
BART2-Bridge	80	(24A)*TGCTGACCGA***GCAAGGGCGAATGCAGAAAATA**(24A)	
BART4-Bridge	90	(29A)*TGCTGACCGA***AGCACACCAGCAGCATCAGGTC**(29A)	

[a] All of the bridge DNAs were designed to recognize the specific miRNA complement published by the Sanger Institute Database (miRBase) [21]. The boldface of the bridge DNA indicates the complementary part of respective miRNAs. The italic of the bridge DNA indicates the counter part of fluorescence probe.

miRNA at a time as a result of limitation of separation resolution. Because miRNAs are a class of short nucleotides with similar length, simultaneous detection of multiple miRNAs is still challenging using electrophoresis-based methods. In past years, Maroney et al., reported a miRNA detection method based on splinted ligation with isotope labeling [27]. Furthermore, Barron's group announced end-labeled free-solution electrophoresis for DNA sequencing by using protein polymers as drag-tags [28]. In this study, we report a novel assay for multiplexed miRNA detection within a single capillary based on an artificial difference in electrophoretic migration.

Considering the biological importance of other EBV miRNAs [29] and the significant difference of miRNAs between normal and EBV-infected NPC tissues [30], the five high abundance miRNAs (BART9, BART7, BART18, BART2, and BART4) were chosen to design the specific bridges that would complement their respective targets (Table 1). The multiplexed detection of EBV miRNAs from NPC cells was achieved using this method. Furthermore, unexpected electrophoretic peak other than the five chosen BART miRNAs were detected from NPC cells by CE-LIF. Therefore, this method may allow scientists to rapidly screen isomiR cost-effectively.



Scheme 1. Schematic representation of tandem adenosine-tailed DNA bridge-assisted splinted ligation and capillary electrophoresis for multiplexed microRNA detection.

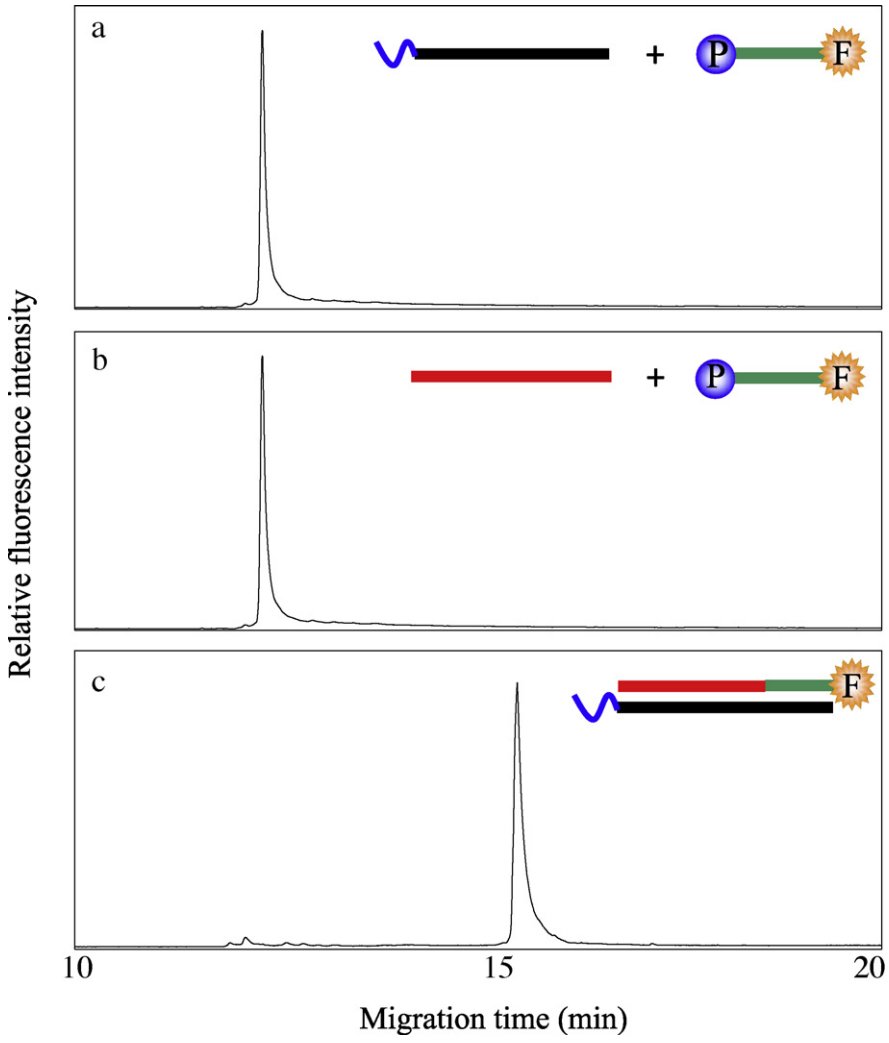


Fig. 1. Electropherograms of control sets. (a) Negative control with no bridges; (b) negative control with no targets; (c) the sample containing the probe, five bridges of different lengths, and synthetic BART7 miRNA. The initial concentrations of the probe, bridges and BART7 miRNA were all 1.0 μM. The reaction products were introduced into the capillary without additional washing. Other conditions are described in Section 2.

2. Materials and methods

2.1. Chemicals and reagents

Urea, poly(ethylene) oxide (PEO) (average M_w 8,000,000 g/mol), polyvinylpyrrolidone (PVP) (average M_w 1,300,000 g/mol), and the chemicals used to prepare the electrolytes (Tris–glycine–acetate) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The customized synthetic HPLC-grade oligonucleotides listed in Table 1 were purchased from Integrated DNA Technologies (San Diego, CA, USA). The sequence of fluorescent labeled probe is designed so as to avoid the hairpin structure and minimize self-duplex ($\Delta G > -2.5$ kcal/mol) by Oligo 7 (Molecular Biology Insights, Inc., USA). Trizol reagent and DNase/RNase-free water were obtained from Invitrogen (Carlsbad, CA, USA). T4 DNA ligase and an RNA extraction kit were purchased from Qiagen (Hilden, Germany).

2.2. Cell culture and RNA extraction

The NPC cells (HK-1) and EBV-infected NPC cells (C666-1) were kindly provided by Prof. Sai-Wah Tsao from the Department of Anatomy at the University of Hong Kong. The cells were cultured in RPMI medium at 37 °C in 5% CO_2 for 3 days. Total RNA was extracted from the HK-1-EBV and HK-1 cell lines using Trizol reagent in accordance with the manufacturer's instructions. DEPC-treated water was used to prepare the RNA stock solutions, and DNase/RNase-free water was used to adjust the synthetic DNA and RNA concentrations. The RNA samples were stored at −80 °C, while all DNA samples were stored at −20 °C.

2.3. Hybridization and splinted ligation

All of the DNA bridges were designed to recognize the specific miRNA complement published by the Sanger Institute Database (miRBase) [31]. A tandem adenosine with a ten-nucleotide interval was located at the 3′ end of the bridges, and the 5′ end was designed to anneal to a short fluorescent probe. For the hybridization reaction of total RNA, 1 μL of the fluorescent probe (0.5 μM), 1 μL of tandem adenosine-tailed bridge DNA (each of 0.1 μM), 0.5 μL of Mg^{II}-free PCR buffer (10× concentration, Roche, USA), and total RNA (0.5 μg) extracted from the nasopharyngeal carcinoma cells were mixed in a PCR microtube. DNase/RNase-free water was then added to the mixture to give a final volume of 5 μL. After vortexing, the microtube was placed in a thermal cycler (Astec, Japan) for miRNA hybridization over a temperature gradient (65–45 °C) for the appropriate time interval (30 s to 12 h). A second hybridization at a constant temperature of 20 °C was used to anneal the fluorescent probe with time interval same as gradient setting. After hybridization, 1 μL of 10× ligation buffer, 0.2 μL of T4 DNA ligase (5 Weiss units) and 3.8 μL of double-deionized water were added,

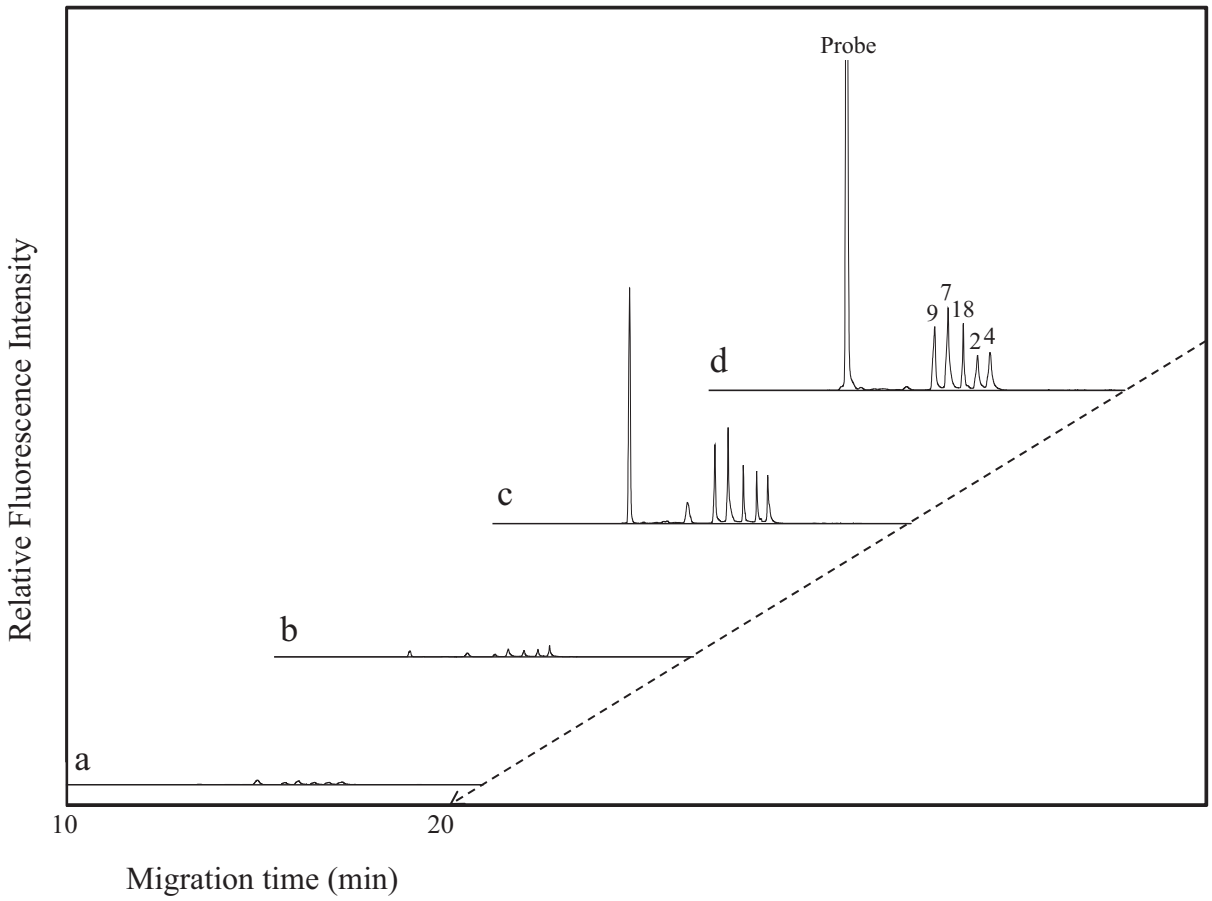


Fig. 2. Effect of sample purification with different ethanol concentrations on the removal of the short nucleic acids (10 nt). The samples were washed four times with the following ethanol concentrations: (a) 40%, (b) 50%, (c) 60%, and (d) 70%. The initial concentrations of the probe, each bridge, and all of the BART oligonucleotides were 0.5 μM, 0.1 μM, and 1.0 nM, respectively. Other conditions are described in Section 2.

and the reaction mixture was incubated at 16 °C for 30 min. When the reaction was complete, ethanol containing 2.5% formamide was added to the tube, and the contents were gently mixed several times by inversion. The longer nucleic acids contained within the tube were then precipitated by centrifugation (10,000 × *g*, 30 min). The reaction products were further purified three times by washing with 60% ethanol without formamide to reduce the buffer content. Finally, residual ethanol/H_2O was removed by a vacuum dryer (Genevac, UK) for 5 min, and the precipitate was resuspended in 5 μL of double-ionized water (Millipore, Billerica, MA, USA). All fluorescent materials, including post-ligation products, were stored at ambient temperature inside a black box.

2.4. CE-LIF

A house-built CE-LIF system was used for miRNA analysis with slight modification from previously setup [26]. 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 within a black box with a high-voltage interlock. The high-voltage end of the separation system was placed inside a plastic bottle for safety. A 532-nm diode-pumped solid-state laser from Changchun New Industries Optoelectronics Tech. (Changchun, China) with a 10-mW output was used for excitation. The excited fluorescence was collected with a 10× objective (numerical aperture = 0.25), and a 532 nm O.D. 6 notch filter (Semrock, Rochester, NY, USA) was arranged after the objective to reject the scattered light. Finally, a 550-nm interference filter and an OG 550 cut-off filter (Edmund, Barrington, NJ, USA) were used to block stray light before the emitted light reached the photomultiplier tube (R3896, Hamamatsu Photonics, Hamamatsu, Japan). The amplified currents were transferred directly through a 10-k(resistor to a 24-bit A/D interface at 10 Hz, controlled using Clarity software (DataApex, Prague, Czech Republic), and the resulting data were stored on a personal computer. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with a 75-μm internal diameter were used for separation. The total capillary length was 50 cm, and the effective length from the detector was 43 cm. Each new capillary was coated with PVP (5% in H_2O) for 1 h to reduce the electroosmotic flow [15]. Prior to sample introduction, the capillary was filled with a copolymer solution from a Teflon tube and pumped with high-pressure nitrogen gas (approximately 200 psi). The copolymer (1.5% PEO, 0.5% PVP) was dissolved in 2× TGA buffer, pH 7.5, (14 mM Tris, 192 mM glycine, and 10 mM acetic acid) [32] and 7 M urea. Briefly, the urea (21 g) was poured into a 50-mL graduated cylinder flask that contained 25 mL of TGA (4×), and double-deionized water was added to the 45-mL mark. After complete dissolution of the urea, the stir bar was removed and double-deionized water was added to the buffer solution to give a total volume of 50 mL. The buffer solution was then transferred to a wide-mouth Erlenmeyer flask containing a magnetic stirring bar. The weighed polymer powder was then gently poured into the buffer solution, and the solution was stirred overnight. The polymer solution was degassed by centrifugation (18,000 × *g* for 10 min.) before electrophoresis. The hybridized nucleic acids were introduced through the cathode end by electrokinetic injection at 200 V/cm for 10 s. The electric field was maintained at a constant 200 V/cm for separation. The high-viscosity polymer solution was

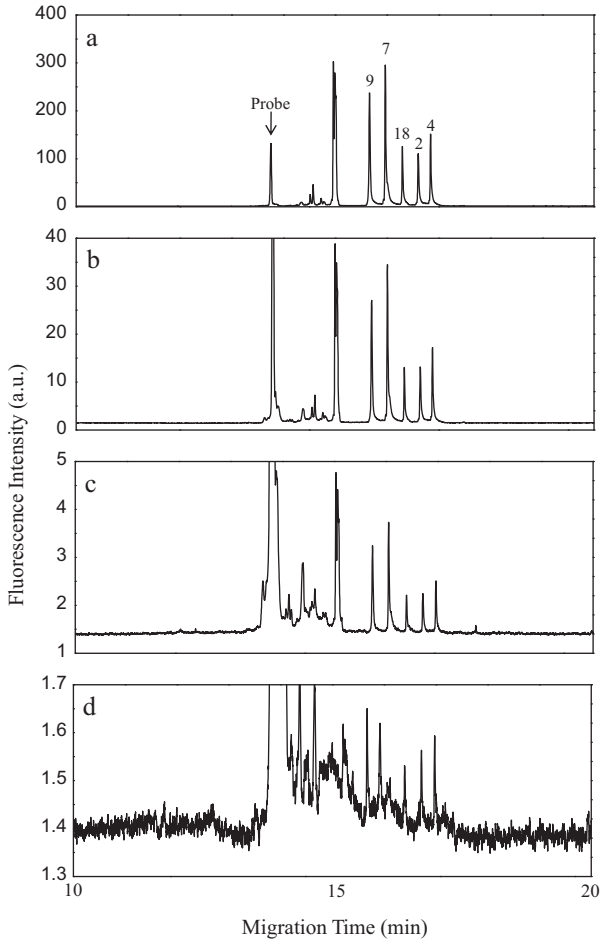


Fig. 3. The electropherograms of the five BART DNAs in serial dilution: (a) 1.0 nM, (b) 0.1 nM, (c) 10 pM, and (d) 1.0 pM. The initial concentrations of the probe and each of the bridges were 0.5 μM and 0.1 μM, respectively. Other conditions are described in Section 2.

pushed out of the capillary by high-pressure nitrogen gas, and, then the capillary was equilibrated with water, 5% PVP, and 2× TGA buffer (pH 7.5) to refresh the capillary wall. The capillary was subsequently filled with copolymer (1.5% PEO, 0.5% PVP) for the next experiment.

3. Results and discussion

3.1. Principle of tandem adenosine-tailed DNA bridge-assisted splinted ligation

MicroRNAs comprise a class of short nucleotides of similar length that include several thousand sequences that cannot be completely separated within a single capillary. We developed a novel method to overcome the complexity of this separation. As shown in Scheme 1, the target miRNAs, tandem adenosine-tailed bridge DNA, and a fluorescent probe were mixed in a microtube. The miRNAs were hybridized to the 3′ end of the bridge DNA that is complementary to miRNAs with varying melting temperatures by gradient temperature hybridization [33]. A second hybridization was carried out after decreasing the temperature to 20 °C to anneal a dye-labeled DNA probe to the 5′ end of the bridge DNA. Following splinted ligation with T4 DNA ligase, the miRNAs were ligated to a fluorescent probe to form longer, double-stranded nucleic acids with the bridge DNA. After ligation and sample washing, the reaction products were introduced into a capillary by electrokinetic injection. The unligated probe/bridge duplex (10 bp) could unwind from the bridge DNA as a result of less hydrogen bonding under the denaturing gel electrophoresis conditions at ambient temperature [26]. Consequently, the single electrophoretic peak represents the free single-stranded DNA probe (10 nt), which migrates faster than all of the other longer ligated products (which are greater than 30 bp in length). As shown in Fig. 1a and b, the negative controls contain only the free fluorescent probe in the electropherogram because there was a lack of one of the components in the double-stranded DNA-dependent ligation. These results also indicate that the products of single-stranded ligations, such as probe-to-bridge or probe-to-miRNA, are negligible. A similar isotope-based splinted ligation method requires phosphatase treatment to reduce the background and poses safety issues due to isotope radiation [34]. Unlike isotope labeling, the clear electrophoretic background caused by on-column denaturing of fluorescent probe was potentially of high enough quality to be used in quantitative applications. On the other hand, the efficiency of splinted ligation for the formation of the phosphodiester bond between the juxtaposed 5′ phosphate and 3′ hydroxyl groups in the BART7-RNA/DNA duplex plays an important role in this design because of the dependence on the activity of the specific ligase [35]. In the positive control set, as shown in Fig. 1c, the T4 DNA ligase provides high ligation efficiency (~80%) for nick coupling of RNA/DNA hybrids (Fig. S1).

3.2. Effects of sample preparation on fluorescence intensity

Because miRNA is a low-abundance biomarker, the hybridization rate is always slow, and thus the reaction takes a long time to reach steady state. In general, overnight incubation has always been required to obtain optimized kinetic and thermodynamic behaviors in heterogeneous hybridizations due to surface-strand density, surface charge, probe length, and substrate flatness effects [36]. In homogeneous hybridization, an increase in the concentration of synthetic nucleic acids facilitates hybridization. However, the synthetic fluorescent probe containing impurities induced high background in the electropherogram, resulting in significant interference to low-abundance target miRNA. To overcome this problem, ethanol washing is used to remove the probe but the most-ligated products were still found in the electropherograms. As shown in Fig. 2, the concentration of the fluorescent probe was minimized by decreasing the concentration of ethanol to 60% (v/v) while the ligated products remained in the microtube based on the ethanol solubility of the nucleic acids of different lengths. The five sharp peaks that had migrated to the far side of the probe appeared in the electropherogram, indicating that the ligated duplexes could be completely separated based on the length differences between the tandem adenosine-tailed bridge DNA.

This result demonstrated that the simultaneous detection of five BART DNAs within a single capillary can be successfully achieved by combining a tandem adenosine-tailed DNA bridge-assisted splinted ligation and CE-LIF. The linear range could cover three orders of magnitude (1.0 nM to 1.0 pM) with a limit of detection (S/N = 3) as low as 190 fM (2.5 zmol), as shown for the synthetic BART9 DNA in Fig. 3. The linearity equation of BART9 is $y = 2.31102 \times 10^{14}x + 421$ with $R^2 = 0.9998$ (Fig. S2). The confused peaks detected between probe and BART9 always increase with the concentrations of five BART DNAs. The peaks mainly contributed by formamide-contained wash step that may unwind the miRNA/bridge DNA duplex thus increase the electrophoretic mobility. On the other hand, the long poly adenosine also undergoes depurination that leads to some degradation of adenosine of bridge DNA [37]. However, more experiments are needed for further demonstration.

3.3. Identification of miRNAs from EBV-infected NPC cells

Epstein-Barr virus-infected nasopharyngeal carcinoma cells were chosen to test the feasibility of our method for clinical applications. HK-1 is a common nasopharyngeal carcinoma cell line that lacks Epstein-Barr virus miRNAs and was used as the negative control. As shown in Fig. 4a, no false positive peak was present in the electropherogram except for the labeled probe, indicating the high specificity of this method even with the complex total human RNA sample. On the other hand, the primary miRNA and/or precursor miRNA always are the interferences for miRNA detection. For example, the stem-loop precursor of BART7 is 86 nt that is much longer than mature BART7 miRNA (22 nt) and contains the BART7 sequence thus interfering the hybridization of mature miRNA. However, the interference caused by primary or precursor miRNA is not the important issue in this design. They may hybrid to bridge DNA but cannot be ligated with fluorescent probe due to the longer nucleotides in 3′-end of mature miRNA sequence thus, blocking the miRNA/probe nick junction. In EBV-infected nasopharyngeal carcinoma cells (C666-1) (Fig. 4b), four of the five expected peaks were present in the electropherogram. By using standard addition (spiked with synthetic BART DNA), the concentrations of BART9, BART7, BART18, and BART4 were determined to be 0.22 nM, 0.10 nM, 27 pM, and 26 pM. Compared to previous results, the transcription level of BART2 should be similar to other four mature BART miRNAs [30,38]. However, we observed that the peak of BART2 dramatically decreased in the electropherogram and was replaced by

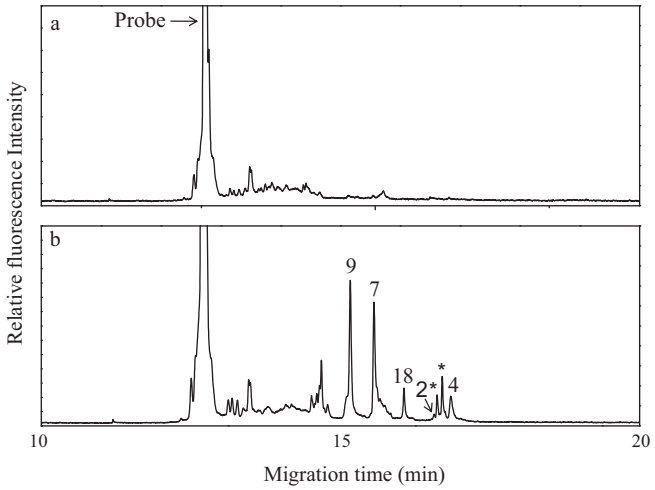


Fig. 4. Electropherograms for miRNA detection in nasopharyngeal carcinoma cells. The samples consisted of the total RNA extracted from the nasopharyngeal carcinoma cell line using (a) non-EBV-infected nasopharyngeal carcinoma cells (HK-1) and (b) EBV-infected nasopharyngeal carcinoma cells (C666-1). The virus-specific miRNAs were validated by spiking the samples with the synthetic oligonucleotides corresponding to the sequences obtained from miRBase. Asterisks mark the possible candidates for the isomiR of BART2. The initial concentrations of the probe and bridges were 0.5 μM and 0.1 μM, respectively and 0.5 μg of extracted total RNA was used. Other conditions are described in Section 2.

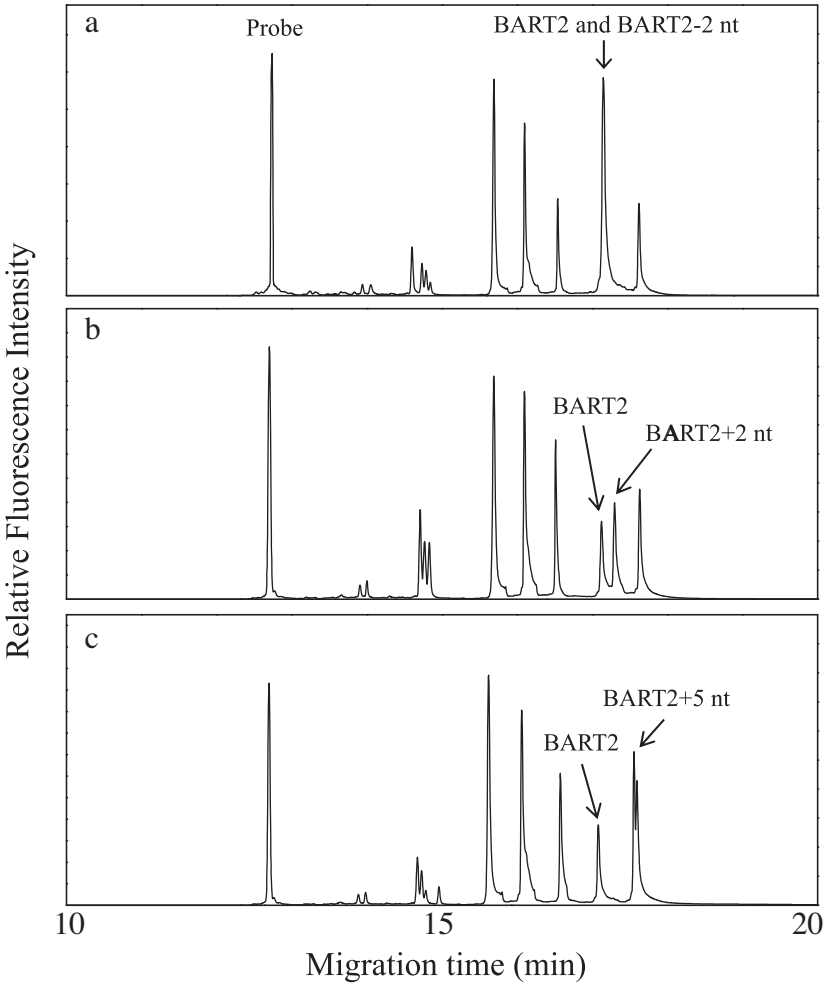


Fig. 5. Alternation of BART2 in 5′ end caused the change of electrophoretic migration. The five BART DNAs (BART9, 7, 18, 2, 4) were spiked in with (a) the 2-nt deletion of BART2, (b) the 2-nt addition of BART2, and (c) the 5-nt addition of BART2. All of the BART DNAs were present at identical concentrations (1.0 nM).

another peak (Fig. 4b). Based on the experimental design, however, this peak should only be observed when the 3′ ends of the miRNAs remain at an invariable length, as published in the database for effective ligation reactions (i.e., coupling with a 5′ phosphate of the probe). Therefore, the shift in migration time may result from an alternate 5′ end on the mature BART2 miRNA that led to a change in the electrophoretic mobility of the ligated products. To confirm this hypothesis, synthesized BART2 DNA with a 5′ end deletion of two nucleotides was used to spike the sample that contained five synthetic BART DNAs. Fig. 5a shows that the 2-nt deletion of BART2 (i.e., BART2-2nt) at the 5′ end was not discernable by 1.5% PEO and 0.5% PVP. Therefore, the BART2-2 nt peak overlapped with that of the original BART2 and caused a peak increase at the BART2 position. A 2-nt addition at the 5′ end of BART2 (BART2 + 2 nt) was used to spike samples of five synthetic BART DNAs. Fig. 5b shows that the spiked BART2 + 2 nt migrated between BART2 and BART4. Finally, we tested a 5′-nt addition at the 5′ end of BART2 (BART2 + 5 nt) to confirm the change in the migration time. The BART2 + 5 nt DNA migrated more slowly than both BART2 and BART2 + 2 nt and was closer to BART4 (Fig. 5c). The migration time shifts caused by the 2-nt and 5-nt additions were probably attributed to the un-pairing between additional nucleotides and poly-adenosine bridge DNA, thus, increasing resistance to electromigration in the presence of a high-viscosity polymer solution. These unexpected electrophoretic peaks suggested the existence of an isomiR due to incomplete cleavage of the 5′ end of primary EBV miRNA [39].

4. Conclusions

This study reported a novel multiplexed assay for detecting miRNAs by combining tandem adenosine-tailed DNA bridge-assisted splinted ligation with denaturing capillary gel electrophoresis with laser-induced fluorescence. This electrophoresis-based method is fast, amplification-free, multiplexed and cost-effective, which are advantages that may lead capillary electrophoresis to become a powerful tool for miRNA detection. Like the DNA sequencing, however, the maximum capacity is limited by reading length of polymer solution. In this study, the tandem adenosine was designed in 10 nt interval, thus, detection of hundred miRNAs in a capillary is possible if high-resolution polymer is introduced [16,40]. Another challenge in this method is – "how to manufacture the long bridge DNA in cost-effective consideration. Because of the length limitation of chemical synthesis of DNA, cloning target-specific sequences into specially designed M13 vectors [41] containing different lengths of poly A sequences or conjugation of the protein polymers [28] to bridge DNA may be one of the possible strategies to overcome this issue. In this work, only one labeled probe is used for all miRNAs detected and having such a small sample requirement, our method is much more economical than other existing commercial products. In the opinion of the authors, if this method combined with automatic capillary array electrophoresis [14,16,22] and integrated microfluidic devices [24,42–44], high-throughput detection (e.g., screening for 100 miRNAs in single capillary or length validation of a few important miRNA markers in capillary array format) with ultrasensitive characteristics should provide a significant contribution to biological studies and disease diagnosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.061.

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