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Accurate MicroRNA Analysis in Crude Cell Lysate by Capillary Electrophoresis-Based Hybridization Assay in Comparison with Quantitative Reverse Transcription-Polymerase Chain Reaction

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Supporting Information

ABSTRACT: Accurate quantitation of microRNA (miRNA) in tissue samples is required for validation and clinical use of miRNA-based disease biomarkers. Since sample processing, such as RNA extraction, introduces undesirable biases, it is advantageous to measure miRNA in a crude cell lysate. Here, we report on accurate miRNA quantitation in crude cell lysate by a CE-based hybridization assay termed direct quantitative analysis of multiple miRNAs (DQAMmiR). Accuracy and precision of miRNA quantitation were determined for miRNA samples in a crude cell lysate, RNA extract from the lysate, and a pure buffer. The results showed that the measurements were matrix-independent with inaccuracies of below 13% from true values and relative standard deviations of below 11% from the mean values in a miRNA concentration range of 2 orders of magnitude. We compared DQAMmiR-derived results with those obtained by a benchmark miRNA-quantitation method—quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR-based measurements revealed multifold inaccuracies and relative standard deviations of up to 70% in crude cell lysate. Robustness of DQAMmiR to changes in sample matrix makes it a perfect candidate for validation and clinical use of miRNA-based disease biomarkers.

Analysis of molecular biomarkers in tissue samples is a promising approach for diagnosis, prognosis, and therapy guidance of cancer and other diseases.[1] Inaccuracies in analysis of molecular biomarkers lead to difficulties with their validation and, consequently, slow down their regulatory approvals for clinical use.[2] Analysis of molecular biomarkers in tissue samples typically involves three major steps: (i) sample collection (tissue excision and storage), (ii) sample preparation (e.g., cell lysis, removal of cell debris, and purification/preconcentration of target molecules), and (iii) sample analysis (quantification of target molecules). Failure to repeat each sample-preparation substep in an accurate/reproducible manner is often the main or sole reason for the inaccuracy or irreproducibility of the final analytical result.[3] Minimizing sample preparation is pivotal to making reliable molecular-biomarker analysis of tissue samples. Ideally, the analysis should be applicable to crude cell lysates.

MicroRNAs (miRNAs) are short (18−25 nucleotides), single-strand, noncoding RNA sequences that function as post-transcriptional regulators of gene expression.[4] Subsets of disease-specific deregulated miRNAs (miRNA fingerprints) have been proposed as molecular biomarkers of cancers,[5−7] Alzheimer's disease,[8,9] heart diseases,[10,11] etc.[12,13] Accurate quantitative analysis of multiple miRNAs is required for validating and using miRNA fingerprints. Accordingly, a significant research effort aims at developing analytical approaches for quantitative analysis of multiple miRNAs.[14−19] With very few exceptions,[18,20] sample preparation includes both cell lysis and RNA extraction. The use of commercially available RNA extraction kits results in varying qualities of isolated RNA and different amounts of residual matrix components in the analytical sample.[21−24] Consequently, the RNA extraction stage introduces systematic errors to quantitation of miRNAs. Ideally, sample preparation should only consist of cell lysis and the analysis should be applicable directly to the lysate. While avoiding errors associated with RNA extraction, the application of miRNA analysis to a crude cell lysate may introduce another type of inaccuracy, which is associated with the interference of

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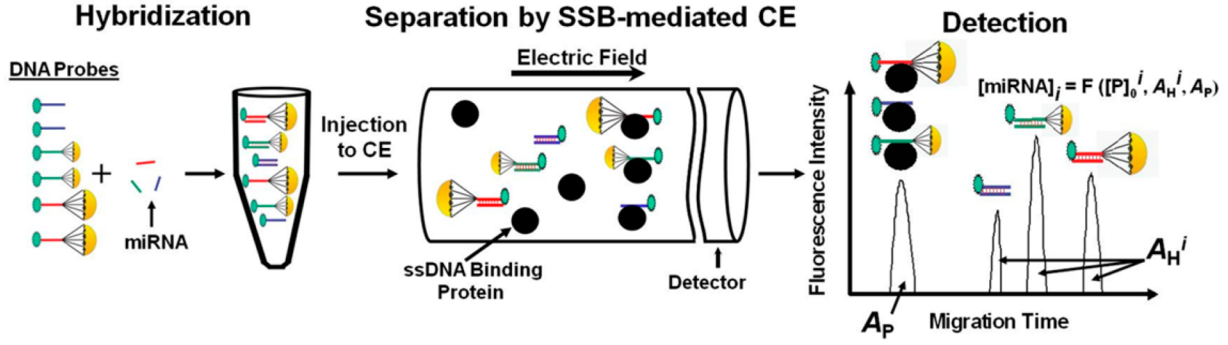

Figure 1. Schematic of DQAMmiR. An excess of DNA probes is employed to specifically bind to target miRNAs forming duplex hybrids. Then the reaction mixture is introduced into CE-instrument to separate unreacted free probes from hybrids and hybrids of different miRNAs from each other. These separations are facilitated by adding single-strand DNA binding protein (SSB) to the run buffer and by modifying probes with drag tags to alter their migration time. The DNA probes are labeled with a fluorophore for laser-induced fluorescence (LIF) detection. The quantification of miRNAs can be achieved by processing the information about the corresponding hybrids in the electropherogram. Adapted from ref 29. Copyright 2011 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.

the cell lysate with the assay components. This interference is important, in particular, for quantitative reverse-transcription polymerase chain reaction (qRT-PCR),[25,26] which is a benchmark method for quantitative analysis of multiple miRNAs.[27,28]

We have recently introduced direct quantitative analysis of multiple miRNAs (DQAMmiR), a capillary electrophoresis (CE)-based hybridization assay (Figure 1).[29] Universal extendable drag-tags were developed to facilitate analysis of multiple miRNAs.[30,31] The limit of detection was improved by designing new detection schemes and sample preconcentration.[32,33] The analysis time was reduced to under 1 h,[34] and single-nucleotide specificity was achieved by utilizing lock nucleic acid (LNA)-containing probes and developing a dual-temperature CE separation regime.[35] In a number of examples, we also demonstrated that sampling the cell lysate did not greatly affect the CE separation pattern.[29,35] These results led us to a hypothesis that DQAMmiR may be robust to changes in the sample matrix and potentially allow highly accurate miRNA analysis in crude cell lysates. Here, we test this hypothesis by investigating the influence of the sample matrix on accuracy and precision of the miRNA analysis. To put our results into a context of the benchmark method, we conducted similar experiments for qRT-PCR. Our results showed that DQAMmiR quantitates miRNA equally well in a pure buffer, RNA extract from the cell lysate, and the crude cell lysate prepared from MCF-7 cultured cells. The DQAMmiR measurements were accurate to within 13% for miRNA concentrations ranging within 2 orders of magnitude. The standard deviations were below 11%. qRT-PCR, in contrast, revealed multifold inaccuracies and standard deviations of up to 70%. These results suggest that the complexity of sample matrix has negligible effect on the analytical performance of DQAMmiR. The robustness of DQAMmiR to the changes in sample matrix indicates that this method can directly quantitate miRNAs in crude samples without the need of bias-prone RNA extractions, making DQAMmiR a very promising candidate for validation and clinical use of miRNA-based disease biomarkers.

MATERIALS AND METHODS

MicroRNA and DNA Probe. MicroRNA (cel-miR-39-3p, 5′-UCA CCG GGU GUA AAU CAG CUU G-3′) and its complementary DNA probe (5′-Alexa488-CAA GCT GAT TTA CAC CCG GTG A-3′) were custom-synthesized by IDT (Coralville, IA, USA). The concentrations of miRNA and DNA probe in stock solutions were determined from light absorbance at 260 nm measured with a Nano-Drop ND-1000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA); molar extinction coefficients were provided by IDT. Working solutions were prepared from stock solutions by dilution. "Actual concentrations" of working solutions were calculated as stock concentrations divided by dilution factors.

Cell Lysate and RNA Extract. MCF-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in the DMEM media (Invitrogen, CA, USA) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested at 50−70% confluency using trypsin/EDTA (0.25%/0.53 mM) and centrifuged at 1500 rpm (375 × g) for 5 min to form a pellet. The pellet was washed twice with PBS. The cells were counted with a hemocytometer. The cells were lysed with 1% Triton in a TAE buffer (50 mM Tris-Acetate, 50 mM NaCl, 10 mM EDTA, pH 8.2) with 10 μM masking RNA (tRNA library from baker's yeast, Sigma-Aldrich, St. Louis, MO, USA) which was used to suppress the degradation of spiked-in miRNA target (cel-miR-39-3p) for the following analysis. Total RNA was extracted by mirVana miRNA isolation kit (Ambion, TX, USA). 1.6×10^6 cells were used to produce 4 mL of cell lysate. The same amount of cells was used to generate 4 mL of RNA extract. Cell lysates and total RNA extract were aliquoted and stored at −80 °C for further analysis.

DQAMmiR. The hybridization step was carried out in a Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Cel-miR-39-3p at varying concentrations was incubated with 100 nM DNA probe. For hybridization, a previously demonstrated temperature program was used.[29] The temperature was increased to a denaturing temperature of 80 °C and then lowered to 37 °C at a rate of 20 °C/min and was held at 37 °C to allow annealing. The optimal hybridization time was derived and used for each sample matrix (see Results and Discussion section). To minimize miRNA degradation the handling of miRNA samples was carried out in a nuclease-free environment.

We used a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Brea, CA, USA) with laser-induced fluorescence detection to carry out separation of the miRNA-

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probe hybrid from the excess of the probe. Bare fused-silica capillaries with an outer diameter of 365 μm, an inner diameter of 75 μm, and a total length of 50 cm were utilized. The distance from the injection end of the capillary to the detector was 40 cm. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, deionized H_2O and run buffer (25 mM Borax, pH 9.2) for 1 min each under a 20 psi pressure. Samples were injected by a pressure pulse of 0.5 psi ×5 s; the volume of the injected sample was 26 nL. Electrophoresis was driven by an electric field of 500 V/cm with positive polarity at the inlet. Fluorescence of the Alexa488 label on the probe was excited by 488 nm light generated with a continuous-wave solid-state laser (JDSU, Milpitas, CA, USA). Electropherograms were analyzed using 32 Karat Software. Peak areas were divided by the corresponding migration times to compensate for the dependence of the residence time in the detector on the electrophoretic velocity of the analyte. Only one miRNA was used in this study; thus, the quantity of miRNA was calculated using the following equation (which is a simplified version of the general equation published elsewhere):[33]

$$[\text{miRNA}] = [\text{hybrid}] = \frac{[\text{P}]_0 A_\text{H}}{A_\text{H} + A_\text{P} q_\text{H}/q_\text{P}} \quad (1)$$

where $[\text{P}]_0$ is the initial concentration of DNA probe, A_H is the peak area corresponding to the miRNA-probe hybrid, A_P is the peak area of the SSB-bound DNA probe, q_H is the relative quantum yield of the hybrid with respect to the free probe, and q_P is the relative quantum yield of the probe in the presence of SSB with respect to the free probe. The relative quantum yields were determined in a set of separate experiments described in the Supporting Information. The original procedure for finding the quantum yields was described in detail elsewhere.[29]

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). TaqMan MicroRNA Reverse Transcription Kit and the stem-loop RT primers for cel-miR-39-3p were purchased from Applied Biosystems (Foster City, CA, USA) and used to synthesize cDNA according to the manufacturer's instructions. The reaction mix was incubated at 16 °C for 30 min, followed by 30 min incubation at 42 °C in the iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The RT products were stored at −20 °C before PCR amplification. Real-time PCR of cDNA from cel-miR-39-3p was performed with an iCycler real-time PCR system (Bio-Rad, CA, USA) using TaqMan-Universal-Master-MixII-no-UNG and TaqMan MicroRNA Assays (both from Applied Biosystems, Foster City, CA, USA). The PCR was started at 95 °C for 10 min followed by 40 amplification cycles each of 15 s at 95 °C and 1 min at 60 °C. 1.5 μL of RT product was used for each 20 μL of PCR reaction.

RESULTS AND DISCUSSION

DQAMmiR involves 3 major steps: (i) hybridization of miRNAs with the probes (the latter taken in excess), (ii) separation of miRNA-probe hybrids from each other and from the unreacted probes, and (iii) quantitative detection of the separated species (Figure 1). In the hybridization step, the complete binding (>90%) of the target miRNAs by the corresponding probes should be reached without appreciable degradation of the miRNAs or the probes. The slower or incomplete binding and the degradation can affect accuracy of miRNA quantitation in DQAMmiR.

The sample matrix can potentially lead to (i) slower or incomplete hybridization becuase of the influence of RNA- and DNA-binding proteins, pH, salts, etc.,[36] and (ii) the degradation of the target miRNAs and DNA probes by nucleases.[37] Thus, for every new sample matrix, the incubation time that is sufficient for the hybridization of >90% of target miRNAs must be found, and insignificant degradation of the target miRNAs and DNA probes during this time must be confirmed.

The adverse effects of the sample matrix on the quality of the hybridization step can be minimized by shortening the incubation time while still ensuring >90% hybridization. The required incubation time can, in turn, be shortened by increasing probe concentrations.[34] Increasing probe concentrations, however, decreases the limit of quantitation (LOQ) of miRNAs because of interferences of signals from the impurities (present in the probes and comigrating with the hybrids) with signals from the hybrids. Therefore, the maximum concentrations of the probes that allow the required LOQ must be chosen first; the incubation time that is sufficient for hybridization of >90% target miRNA must be determined after that.

In this work, we used a single nonhuman miRNA, cel-miR-39-3p, as target miRNA and, accordingly, a single fluorescently labeled complementary to cel-miR-39-3p DNA as a hybridization probe. Cel-miR-39-3p is commonly used as a miRNA mimic in analyses of human miRNA.[38] We set LOQ at a level of 0.1 nM of cel-miR-39-3p. The impurity level in the DNA probe was measured (see Figure S1), and the maximum DNA probe concentration that allowed us to achieve the set LOQ was found to be 100 nM. This probe concentration was used in all further experiments.

With the probe concentration being defined, we studied the influence of sample matrix on the incubation time required for hybridization of >90% of target miRNA. Three matrices were investigated: a pure TAE buffer (50 mM Tris-acetate, 50 mM NaCl, 10 mM EDTA, pH 8.2), an RNA extract from the cell lysate of MCF-7 cultured cells (obtained with a commercial miRNA isolation kit), and the crude lysate from the MCF-7 cells. Cel-miR-39-3p, which is foreign for MCF-7 cells, and its fluorescently labeled complementary DNA probe were spiked into the 3 matrices at final concentrations of 10 and 100 nM, respectively. The incubation time was varied between 10 and 150 min. CE separation and hybrid quantitation with eq 1 were then conducted. Hybrid concentrations as functions of incubation time for the 3 matrices are shown in Figure 2. Incubation times below 30 min were insufficient for reaching hybrid concentration of 9 nM (90% hybridization of 10 nM miRNA). Upon reaching its maximum hybrid concentration does not change significantly for the pure buffer but gradually decreases for the RNA extract and the cell lysate. The decrease is likely associated with the degradation of the target miRNA which is in agreement with the fact that the decrease rate is significantly higher for the cell lysate. For our specific case, incubation times in a range of 30−60 min were suitable for all 3 sample matrices—they ensured miRNA integrity and >90% hybridization. For further experiments we chose incubation time of 45 min as the optimum one.

As we mentioned above, the accuracy of miRNA quantitation also depends on the integrity of the DNA probe. We, thus, studied probe degradation in the 3 matrices. 100 nM DNA probe was spiked into each matrix along with fluorescein as an internal standard and incubated for varying times before

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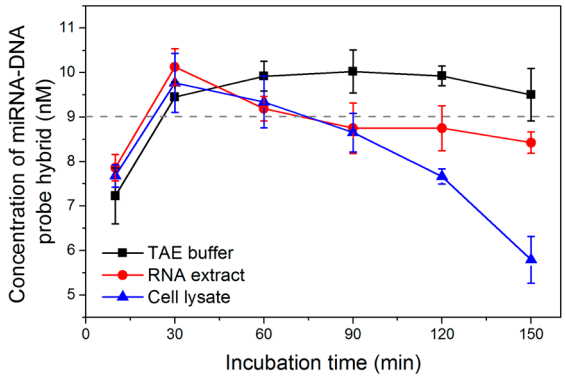

Figure 2. Effect of sample matrix on miRNA hybridization. Both 10 nM miRNA and 100 nM DNA probe were spiked into the 3 matrixes and incubated for varying times; after that, the hybrid was separated from the unreacted probe by CE and hybrid concentration was calculated with eq 1. The dashed line shows the level of 90% hybridization of the spiked 10 nM miRNA. Error bar represents standard deviation of three repetitions.

sampling for CE separation. We found that expectedly probe degradation was very slow in all the matrices (Figure 3). Even

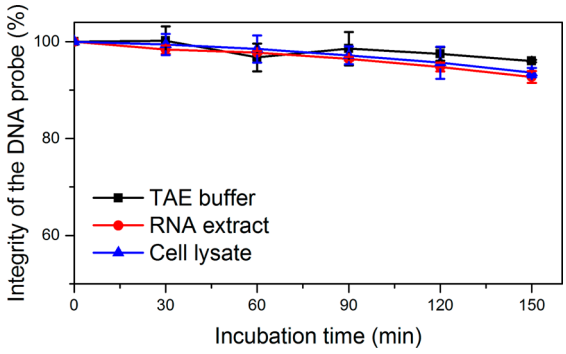

Figure 3. Integrity of the DNA probe in each matrix over time. 100 nM DNA probe and 50 nM fluorescein (as internal standard) were spiked into the 3 matrices, following incubation for varying times and analyzed by CE. The integrity of the DNA probe was calculated by using the relative peak area of the DNA probe. The integrity of the DNA probe without incubation was set as 100%. Error bar represents standard deviation of three repetitions.

when incubated in the cell lysate for 2-h, 90% of the probe was still intact suggesting that probe degradation should not significantly affect accuracy of miRNA quantitation by DQAMmiR. If significant probe degradation is observed, the use of masking DNA can suppress this degradation.[39] For the purpose of this work, the above experiments confirmed that the hybridization step with 45 min incubation is sufficient for >90% hybridization and guarantees no significant degradation of the target miRNA or DNA probe.

We further studied the performance of DQAMmiR in different sample matrices by measuring spiked-in miRNA target, cel-miR-39-3p in a range of concentrations. The range of miRNA concentrations was set 0.1−10 nM, while the probe concentration was 100 nM in all experiments. DQAMmiR electropherograms obtained for the 3 matrices (Figure 4 and Figure S2) indicate that there was no significant matrix-associated effect on CE separation. Quantifiable peaks of the miRNA-probe hybrid were successfully produced in all matrices for all miRNA concentrations (0.1−10 nM).

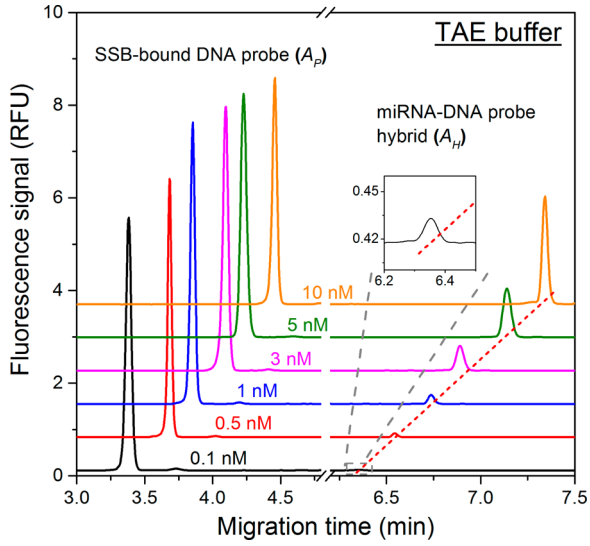

Figure 4. Electropherograms of DQAMmiR measurements in the TAE buffer. Concentration range of miRNA is 0.1−10 nM. The inset shows the zoom-in of the hybrid peak while [miRNA] = 0.1 nM for clarity.

The results of DQAMmiR experiments were compared with the results of the qRT-PCR assay, which is a benchmark method for miRNA quantification. According to standard procedures, a standard curve is required for qRT-PCR for absolute quantification.[27,28] Here we built the standard curve using standard samples prepared by serial dilution in TAE buffer (Figure S3). The concentration range of standards was selected to cover the range of interest in this study (0.1−10 nM). The reason why the standard curve must be built for the pure buffer is that in a real case scenario, RNA extract and cell lysate from the real sample contain endogenous target miRNA. Bar graphs in Figures 5 compare the concentrations of miRNA measured by DQAMmiR and qRT-PCR with actual concentrations for the three sample matrices. Note that the vertical axis is in the logarithmic scale. In the TAE buffer (Figure 5a), DQAMmiR could perfectly recover miRNA concentrations in their whole range with inaccuracy below 10% and relative standard deviation (RSD) below 9%. The results of qRT-PCR showed slightly worse accuracy (inaccuracy of 22%) and precision (RSD of 34%), specifically at lower miRNA concentrations. For miRNA samples in the RNA extract and cell lysate, the differences between DQAMmiR and qRT-PCR were drastic (Figures 5b and c). Measurements by DQAMmiR were still accurate (maximum inaccuracy of 13%) and precise (maximum RSD of 11%). In contrast, 5-fold inaccuracies and RSD of up to 70% were observed in the qRT-PCR results. DQAMmiR proved to be perfectly robust to changes of the sample matrix, while qRT-PCR showed the lack of robustness.

In contrast to DQAMmiR, qRT-PCR requires a standard curve for absolute quantification. This requirement makes qRT-PCR an indirect method[15] and can result in profound inaccuracy and imprecision. In this study, the differences in accuracy between the 3 matrices suggest that the main source of this inaccuracy was from the mismatch of the matrices, the standard curve was built for miRNA spiked in the pure buffer. (We would like to emphasize again that in a real case scenario, it is impossible to build a calibration curve in the matrix of a biological sample as it already contains the target miRNA and, thus, does not allow one to control the concentration of target

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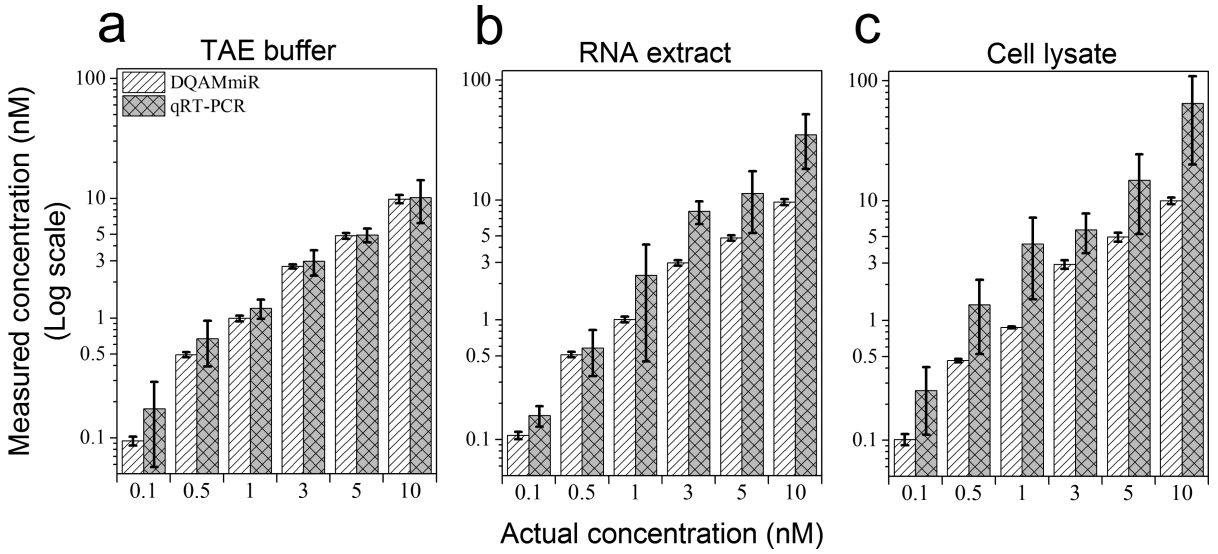

Figure 5. Comparison of miRNA recovery in DQAMmiR and qRT-PCR-based for (a) TAE buffer, (b) RNA extract, and (c) cell lysate. Error bar represents the standard deviation of three independent measurements.

miRNA.) In contrast to the pure TAE buffer, the crude cell lysate contains molecules (proteins, nucleases, and RNA/DNA, etc.), which can influence the activity of the two enzymes (reverse transcriptase and DNA polymerase) employed in qRT-PCR.[25] Typically, preanalytical purifications, such as RNA isolations, are used to purify cell lysates and reduce this interference from the matrices, however, our investigation of samples with RNA extract as a matrix indicated that the extraction kits might not be able to completely remove these interfering contents from crude samples. Moreover, these additional processes for sample preparation could also introduce unpredicted variances in the analyte amount, resulting in an unreliable miRNA expression profile.[40] In contrast, the hybridization in the DQAMmiR assay is an enzyme-free reaction, the conditions of which could be easily altered and optimized without disturbing its quantitative characteristics in crude matrices.

To conclude, the excellent accuracy and precision of DQAMmiR in cell lysate confirmed that DQAMmiR is applicable to crude cell lysate and is very robust to changes of sample matrices. Therefore, DQAMmiR can facilitate highly accurate analyses of crude clinical samples without additional sample preparation. Such analyses, in turn, will facilitate validation of "miRNA fingerprints" for disease diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b00707.

Results of quantum yield measurements, electropherograms of DQAMmiR measurements in RNA extract and cell lysate, standard curve of qRT-PCR, and measured results by two methods (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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