Improvements to Direct Quantitative Analysis of Multiple MicroRNAs Facilitating Faster Analysis

ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2013

Impact Factor: 5.64 · DOI: 10.1021/ac402812f · Source: PubMed

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Improvements to Direct Quantitative Analysis of Multiple MicroRNAs Facilitating Faster Analysis

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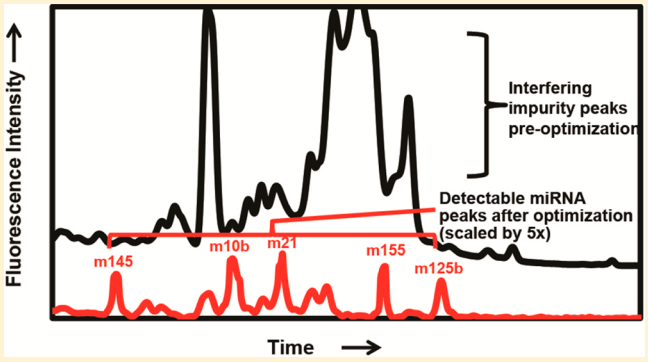
Supporting Information

ABSTRACT: Studies suggest that patterns of deregulation in sets of microRNA (miRNA) can be used as cancer diagnostic and prognostic biomarkers. Establishing a "miRNA fingerprint"-based diagnostic technique requires a suitable miRNA quantitation method. The appropriate method must be direct, sensitive, capable of simultaneous analysis of multiple miRNAs, rapid, and robust. Direct quantitative analysis of multiple microRNAs (DQAMmiR) is a recently introduced capillary electrophoresis-based hybridization assay that satisfies most of these criteria. Previous implementations of the method suffered, however, from slow analysis time and required lengthy and stringent purification of hybridization probes. Here, we introduce a set of critical improvements to DQAMmiR that address these technical limitations. First, we have devised an efficient purification procedure that achieves the required purity of the hybridization probe in a fast and simple fashion. Second, we have optimized the concentrations of the DNA probe to decrease the hybridization time to 10 min. Lastly, we have demonstrated that the increased probe concentrations and decreased incubation time removed the need for masking DNA, further simplifying the method and increasing its robustness. The presented improvements bring DQAMmiR closer to use in a clinical setting.



MicroRNAs (miRNAs) are short noncoding RNAs that are involved in regulation of gene expression. Abnormal expression of miRNAs has been observed in many diseases, including cancer.[1−4] Various types of cancer and stages of malignancy have been associated with deregulation of specific subsets of miRNA. It has been suggested that detection of such miRNA deregulation patterns, or "miRNA fingerprints", rather than an individual miRNA, can be used for cancer diagnostics or as predictive and prognostic biomarkers.[3,5] Establishment of a viable diagnostic technique requires availability of a miRNA quantitation method that satisfies the following criteria: (i) miRNA detection must be sequence-specific and resistant to sequence-related quantitation biases; (ii) the method must offer limits of quantitation sufficient for analysis of low-abundance miRNAs; (iii) the method must allow simultaneous analysis of multiple miRNAs within a "fingerprint" set; and (iv) the analysis procedure must be rapid and robust.

Most current miRNA detection methods, such as quantitative reverse transcription polymerase chain reaction, microarrays, surface plasmon resonance, and next-generation sequencing, require amplification or chemical modification of miRNAs.[6−9] While these methods are invaluable for semi-quantitative screening of miRNA expression profiles, their rates of amplification and modification of nucleic acids of different nucleotide sequences often vary even for molecules of similar length. These sequence-related biases skew the representation of individual sequences in quantitative analyses, making the accuracy of such indirect methods unsuitable for clinical applications.[10−12] Several direct methods for detection of multiple miRNAs have been suggested, but most are still in early stages of development and require significant improvement to be feasible in clinical settings.[13−20] In contrast, direct quantitative analysis of multiple miRNAs (DQAMmiR) is a method that has been developed specifically with a focus on clinical applications. DQAMmiR is an assay that detects miRNA through hybridization with complementary fluores-

Received: September 4, 2013
Accepted: September 29, 2013
Published: September 29, 2013

cently labeled DNA probes. Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection is used to separate and quantitate individual miRNA–DNA hybrids and the excess of DNA probes. Separation between individual miRNA–DNA hybrids is achieved by covalently attaching different mobility-shifting drag tags to the hybridization probes.[20,21] Separation of the nonhybridized DNA probes from the hybrids is facilitated by single-strand DNA binding protein (SSB) present in the CE running buffer. SSB binds the nonhybridized DNA probes, shifting their mobility and allowing for their separation from the hybrids.[20]

As a hybridization assay, DQAMmiR is highly specific and does not suffer from sequence-related quantitation biases. The method was previously used to detect as few as 1000 copies of miRNAs (100 pM), and our recently introduced universal procedure for development and conjugation of drag tags theoretically allows simultaneous analysis of up to 25 different miRNAs.[21,22] The analysis of 5 miRNAs has been practically demonstrated.[21] DQAMmiR was also shown to be effective in detection of miRNAs within complex biological mixtures, such as cell lysates.[20] However, a few technical limitations have so far precluded the use of DQAMmiR in clinical settings. The sensitivity of the method strongly depends on the quality of DNA probes. Presence of fluorescent impurities, such as DNA degradation products which are too short for SSB-binding or byproducts of the drag tag conjugation reaction, interferes with detection of miRNA–DNA hybrids and significantly undermines the method's limit of quantitation. After synthesis and drag-tag conjugation, lengthy multistep purification is required to produce a DNA hybrid preparation of a sufficient quality. Moreover, the interfering fluorescent impurities in DNA hybrid preparations tend to reappear over time due to sample degradation. Diligent storage procedures mitigate this issue but are often not sufficient, prompting for additional poststorage purification steps. Failure to eliminate the interfering fluorescent impurities imposes a limitation on the maximum concentration of DNA probes that can be used in the assay, often restricted to no more than 50 nM. As a consequence, the incubation time of the DNA probes with miRNAs increases significantly, usually requiring an overnight hybridization time (defined as the time required to hybridize over 90% of miRNA targets).[22] While increasing the temperature can increase the rate of hybridization, this tool is not very efficient since hybrid stability decreases with raising temperature. With the CE part of the analysis requiring less than 10 min, such a prolonged period of hybridization presents a major shortcoming of the method. Furthermore, the long incubation time of the DNA probes, especially within complex biological samples, necessitates the use of antinuclease masking DNA. Addition of masking DNA requires careful sequence and concentration optimization to prevent nonspecific hybridization, further reducing the robustness of DQAMmiR. Thus, the long incubation is perhaps the main obstacle in adaptation of DQAMmiR to the clinical setting.

In this work, we introduced a set of improvements to DQAMmiR that address the described technical limitations. First, we have devised a quick and simple poststorage purification procedure that efficiently eliminated the interfering fluorescent impurities from DNA probe preparations. This filtration-based approach decreased the concentrations of interfering impurities by a factor of ~25 to the levels of <0.005% of the probe concentrations. This significantly alleviates the limitation on maximum probe concentrations used in the assay. Second, we have optimized the assay concentrations of the DNA probe to decrease the hybridization time to 10 min. This improvement allowed completion of a single DQAMmiR measurement in approximately 20 min, the shortest analysis time for any available method for multiple-miRNA quantitation. Lastly, we demonstrated that the increased probe concentrations and decreased incubation time removed the need for masking DNA, further simplifying the method and increasing its robustness. The presented modifications bring DQAMmiR closer to use in a clinical setting, making it, arguably, the most suitable method for miRNA-based diagnostics.

RESULTS AND DISCUSSION

The presence of fluorescent impurities in the DNA probe preparations significantly undermines DQAMmiR's limit of quantitation. As evident from Figure 1, top trace, even after dual reverse-phase HPLC purification, the sample contained a significant amount of impurities. At the total probe concentration of 500 nM, the peaks associated with fluorescent impurities completely obscured the presence of a hybrid peak corresponding to 100 pM of miRNA. Conjugation of the DNA probes to a peptide drag tag, through a recently reported procedure, further increased the level of interfering impurities in the preparation (Figure 1, middle trace).[22] The effect of these impurities could not be eliminated by optimization of CE separation conditions, as electrophoretic resolution of short oligonucleotides in free solution is, in general, difficult due to their similar charge-to-length ratios. Elimination of the observed impurities was required in order to ensure sufficient limits of miRNA quantitation. At the same time, to be viable in a clinical setting, the method of poststorage purification must be fast, inexpensive, and accessible. According to the observed CE migration patterns, the interfering impurities are most likely composed of truncated DNA probe fragments. As a result, we have attempted to remove these impurities through molecular weight filtration. After testing a number of strategies and products, we have determined that the Amicon Ultracel filter

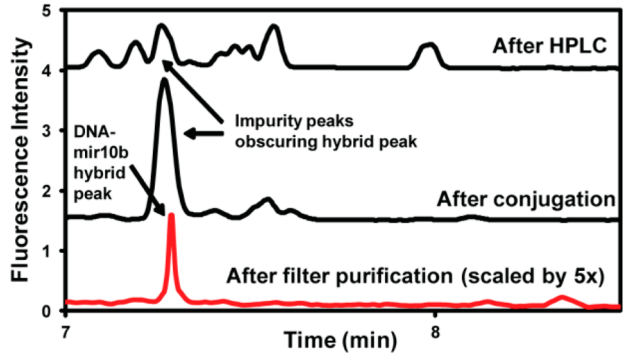


Figure 1. Impurity peaks in a 500 nM miR-10b DNA probe sample at different steps of purification. Each sample also contained 100 pM miR-10b. Top trace: miR-10b DNA probe purified with dual reverse-phase HPLC. The DNA probe-miR-10b hybrid peak cannot be observed due to its overlapping impurity peaks. Middle trace: miR-10b DNA probe conjugated to a 15aa peptide. Impurity peaks once again obscure the hybrid peak. Bottom trace: miR-10b-15aa probe that has been purified with the molecular weight filter; the trace scaled up by a factor of 5. The absence of impurity peaks allows for the detection of the hybrid peak (red trace), which is the only observable peak. The running buffer contained 50 nM SSB for each run.

dx.doi.org/10.1021/ac402812f | Anal. Chem. 2013, 85, 10062–10066

from Millipore with a 10K Dalton pore size achieves sufficient level of purification within a single filtration step. This filter was able to retain full-sized conjugated DNA probes, while allowing the impurities to pass through. Filters with smaller and larger molecular cutoff values were also tested, but the obtained results suffered from decreased efficiency and decreased yield, respectively. Incorporating this poststorage purification step decreased the overall yield of DNA probe purification by 15%, a loss which, we believe, is well worth the improved quantitation limit. The inexpensive filters can be easily adapted for use by clinical technicians and only require the use of a common centrifuge. As shown in Figure 1, bottom trace, the 100 pM miRNA peak can be easily distinguished when a purified DNA probe preparation is used for its detection.

With elimination of fluorescent impurities, the limit on maximum assay concentration of the DNA probe is significantly alleviated. This limit was defined as the highest probe concentration at which the presence of interfering fluorescent impurities did not interfere with detection of 100 pM miRNA samples. As a result, the probe concentration can be increased to facilitate faster hybridization with its miRNA target. In previous implementations of DQAMmiR, hybridization time required a lengthy incubation of up to 8 h. Our goal was to decrease the hybridization time to be comparable with time of capillary preconditioning steps, which take approximately 10 min. This way, sample hybridization can be performed concurrently with capillary preconditioning, followed by a 10-min CE analysis step, reducing the overall time of a single DQAMmiR measurement to approximately 20 min. Reduction of the hybridization time below 10 min would not significantly shorten DQAMmiR analysis time but would raise the analysis cost by increasing consumption of the fluorescent DNA probes. To find the optimum DNA probe concentration, at which the intended 10-min hybridization time was achieved, we incubated a 100 pM miRNA sample with increasing concentrations of its probe, starting from 1 nM. Efficiency of hybridization was assessed by DQAMmiR measurements of a mir-21 sample of a known concentration. As shown in Figure 2, the hybridization time steadily decreased with increasing probe concentrations, starting from 5 h for 1 nM probe and reaching the goal hybridization time of 10 min at a concentration of 500 nM. Molecular-size filtered preparations of the probes used at this concentration did not show a significant presence of interfering fluorescent impurities. The introduced improvements, thus, allowed us to increase the limit on maximum assay concentration of the DNA probe from 50 to 500 nM.

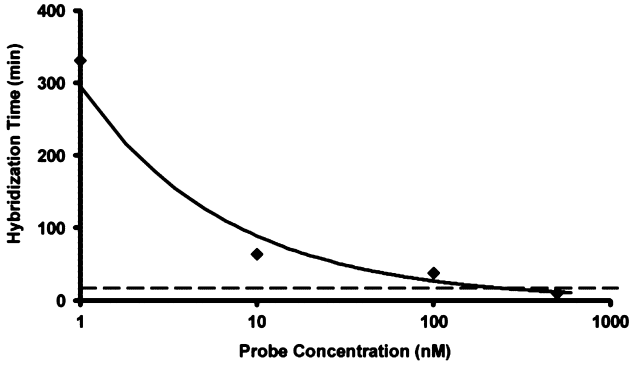


Figure 2. Optimization of DNA probe concentration to achieve target hybridization time. 100 pM of miR-21 was incubated with increasing concentrations of its respective DNA probe until a 10 min hybridization time (indicated by the dashed line) was achieved. Hybridization time steadily decreased from 5 h with 1 nM down to the goal of 10 min, which was achieved with 500 nM DNA probe.

One of the great advantages of DQAMmiR is its ability to be applied directly to complex biological samples. Previous applications of DQAMmiR to cell lysate-containing mixtures, with the prolonged incubation times, required the use of masking DNA to suppress nuclease-facilitated degradation of the DNA probes. In order to prevent the formation of nonspecific probe/masking DNA duplexes and to avoid interference of masking DNA with formation of probe/target duplexes, both the sequence and the concentration of the masking DNA required careful consideration. Furthermore, the use of high concentrations of masking DNA significantly raised DQAMmiR analysis cost. With the achieved reduction in hybridization time, however, the need for masking DNA was re-examined. We studied the kinetics of DNA probe degradation within the context of a bacterial cell lysate. The choice of a bacterial cell lysate was intentional, as it displayed a higher extent of probe degradation when compared to mammalian cell lysate (Figure S1, Supporting Information). The extent of DNA probe degradation, taken at 500 nM concentration, was assessed on the basis of the decrease of the DNA peak area from CE analysis. No SSB or masking DNA was used. As seen in Figure 3, 5 h incubation, without masking DNA, resulted in

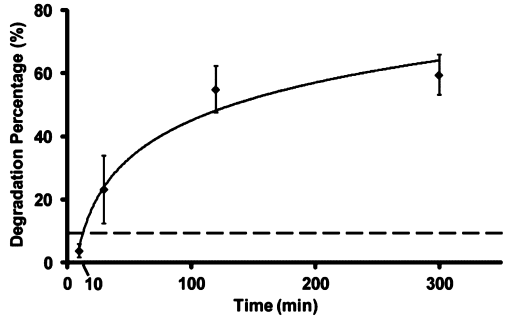


Figure 3. Degradation of DNA probe by cell lysate over time. 500 nM of miR-21 DNA probe was incubated with a bacterial cell lysate over various time periods. Using CE, degradation of the probe was determined on the basis of the decrease in DNA peak area. Minimal degradation was observed after a 10 min incubation. The dashed line represents the degradation level for 5 h incubation with the presence of masking DNA.

an unacceptable 60% level of probe degradation. Ten minute incubation, however, resulted only in 4% degradation, which was lower than the amount of degradation at 5 h incubation with added masking DNA (Figure 3, dashed line). Furthermore, since SSB binds DNA nonspecifically, the removal of masking DNA allowed a lower SSB concentration to be used. In addition, the degradation products in a sample with short incubation time would most likely remain sufficiently long to be eliminated by SSB-binding, making them less detrimental to measurement accuracy. These results suggests that not only do our modifications to DQAMmiR remove the need for the masking DNA, but also they improve the robustness of the method. To test the modified DQAMmiR method, we incubated 5 different miRNA-specific DNA probes, each at 500 nM, with their respective miRNA for 10 min in the presence of bacterial cell lysate, without addition of masking DNA. The DNA probe preparation was subjected to molecular

dx.doi.org/10.1021/ac402812f | *Anal. Chem.* 2013, 85, 10062−10066

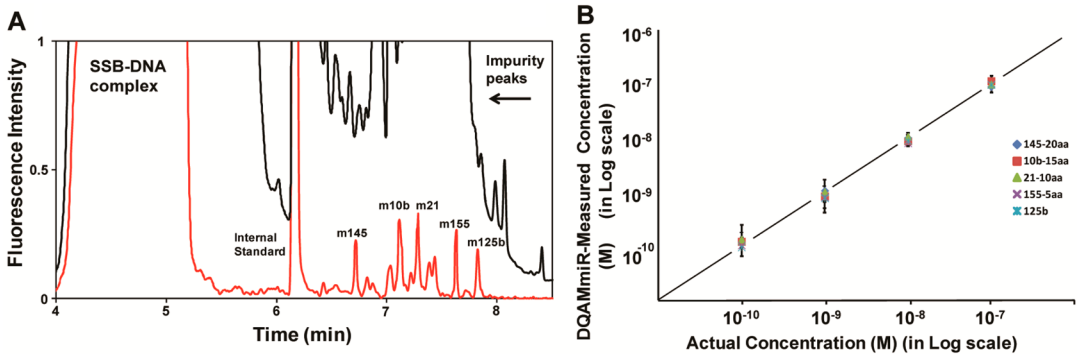


Figure 4. Separation, detection, and quantitation of 5 miRNA species. 100 pM to 100 nM of miR-125b, 155, 21, 10b, and 145 were incubated with 500 nM of their respective DNA–peptide probes (Table S4, Supporting Information) in the presence of cell lysate for 10 min. (A) The detection of 100 pM of 5 DNA–miRNA hybrid peaks, along with the internal standard, fluorescein, and the SSB–DNA complex peak using the optimized protocol (red trace). For comparison (black trace), the previous DQAMmiR protocol (overnight incubation, with masking DNA, no filtering) was also used to detect 100 pM of 5 miRNA species. The large impurity peaks overlapping the hybrid peaks (black trace) prevented the detection and quantitation of the miRNAs. All hybrid peak areas were normalized by their respective quantum yields (Tables S1–S3, Supporting Information). The full extent of interfering fluorescent impurities in the previous DQAMmiR protocol can be viewed in Figure S2, Supporting Information. (B) Quantitative analysis of 100 pM to 100 nM of all 5 miRNA (miR-125b, 155, 21, 10b, and 145), with error bars included. Concentrations of miRNA were validated by light absorbance at 260 nm. Each data point is based on 3 measurements. Error bars represent 3 standard deviations.

weight filtration prior to experiments which significantly reduced the impurity peaks in comparison to the previous DQAMmiR protocol (Figure 4A). The increased concentrations of the DNA probe did not reveal significant interfering impurities and did not affect our ability to separate and detect all 5 hybrid peaks. The small peaks surrounding the target miRNA peaks are most likely due to presence of hybridization probes with incomplete peptide drag tags. Their presence, however, does not significantly interfere with our measurements. This allowed us to accurately quantify the miRNAs (Figure 4A) in a short time, while maintaining the sensitivity and multiplexing ability of the method. The measurements were successfully repeated with varying concentrations of the 5 miRNA targets, between 100 pM and 100 nM, showing that the dynamic range of the method spans over 3 orders of magnitude (Figure 4B). This shows that we were able to significantly improve on the deficiencies of the DQAMmiR method, while retaining all of its advantages.

■ CONCLUSIONS

We have presented a set of modifications to the DQAMmiR method that make it more compatible with applications in a clinical setting. We have developed a quick and simple DNA probe purification procedure that significantly reduces the negative effect of interfering fluorescent impurities in the sample. We have optimized the hybridization probe concentration to reduce the overall assay time to approximately 20 min. The reduction in assay time also removed the need for masking DNA. The introduced modifications make DQAMmiR a much faster and more robust method for quantitation of multiple miRNAs. In this present implementation, DQAMmiR is, arguably, the most suitable method for miRNA-based diagnostics of cancer.

■ MATERIALS AND METHODS

Materials. All DNA and miRNA for hybridization assays were custom synthesized by IDT (Coralville, IA, USA). Concentrations of DNA stock solutions were determined by dividing OD_{260} values, obtained using UV-spectrophotometry, by the manufacturer-provided extinction coefficient. *E. coli* single-strand DNA binding protein (SSB) was purchased from Epicenter Biotechnologies (Madison, WI, USA). All other materials were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Sequences of all miRNA, DNA, and peptide molecules can be found in Table S4, Supporting Information. Preparation of cell lysates is described in the Supporting Information.

CE-LIF. All experiments were performed using a P/ACE MDQ CE instrument (Beckman-Coulter, Fullerton, CA, USA) equipped with an LIF detector. A 488 nm line of continuous wave solid-state laser (JDSU, Santa Rosa, CA), with 1 mW effective output was utilized to excite fluorescence. Fluorescence signal was detected at 520 nm wavelength. 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, pH 9.2, with or without 50 nM SSB. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, deionized H_2O, and running buffer for 1 min each. Samples were injected at the positive end by a pressure pulse of 0.5 psi for 5 s; the volume of the injected sample was ~6 nL. Electrophoresis was driven by an electric field of 500 V/cm with normal polarity and coolant controlled temperature maintained at 15 °C. 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 species. All areas were normalized by dividing them by the area of the internal standard, fluorescein. Concentrations of miRNA were determined using equation S1, Supporting Information, which was derived in our previous work.[20]

Hybridization Conditions. Hybridization was carried out in Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Various concentrations of the miR-21 DNA probe were incubated either with or without 100 pM miR-21 for various time periods in incubation buffer (50 mM Tris-Ac, 50 mM NaCl, 10 mM EDTA, pH 8.2). Twenty nM of fluorescein was included in each run as an internal standard. Temperature was increased to a denaturing 80 °C and then lowered to 37 °C at a rate of 20 °C/min and was held at 37 °C to allow

dx.doi.org/10.1021/ac402812f | *Anal. Chem.* 2013, 85, 10062–10066

annealing. To minimize miRNA degradation, a nuclease-free environment was used while handling miRNA samples.

Detection of Multiple miRNA. To allow separation of the 5 hybrid peaks, peptide drag tags of varying size were conjugated to the DNA probes via a thioether bond. The conjugation reaction, which is described in our previous work,[21] occurs between a thiol group on the 5′ end of the DNA probes and a maleimide group on the N terminus of each peptide. All maleimide modified peptides were synthesized by Canpeptide (Pointe-Clare, QUE, Canada).

Removal of Impurities from DNA–Peptide Probes. After conjugation and purification, each DNA–peptide probe was centrifuged with an Amicon Ultracel 10K filter (Millipore, Billerica, MA) for 20 min at 5500 rpm to remove impurities. The retentate, which contained the probe, was collected and further centrifuged with an Amicon Ultracel 3K filter (Millipore, Billerica, MA) for 30 min at 5500 rpm to reduce the volume to make a stock solution.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The work was funded by the Natural Sciences and Engineering Research Council of Canada.

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