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Universal Drag Tag for Direct Quantitative Analysis of Multiple MicroRNAs

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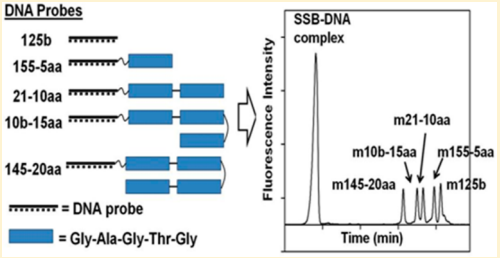
Supporting Information

ABSTRACT: Using microRNA (miRNA) as molecular markers of diseases requires a method for accurate measurement of multiple miRNAs in biological samples. Direct quantitative analysis of multiple miRNAs (DQAMmiR) has been recently developed based on a classical hybridization approach. In DQAMmiR, miRNAs are hybridized with excess fluorescently labeled complementary DNA probes. Capillary electrophoresis (CE) is used to separate the unreacted probes from the hybrids and the hybrids from each other. The challenging separation is achieved by using two types of mobility modifiers. Single-strand DNA binding protein (SSB) is added to the running buffer to bind and shift the single-stranded unreacted probes from the double-stranded hybrids. Different drag tags are built into the probes to introduce significant differential mobility between their respective hybrids. For the method to be practical it requires a universal extendable drag tag. Polymers are a logical choice for making extendable drag tags. Our recent theoretical work suggested that short peptides could provide a sufficient mobility shift to facilitate DQAMmiR. Here, we experimentally confirm this prediction in the analysis of five miRNAs: mir10b, mir21, mir125b, mir145, and mir155. We conjugated four fluorescently labeled DNA molecules with peptides of 5, 10, 15, or 20 neutral amino acids in length; the fifth probe was peptide-free. The peptide tags showed no interference with SSB binding to the probes and facilitated separation of the five hybrids. The mobilities of the five hybrids were used to refine the previously suggested theory. The analysis was performed in both a pure buffer and in cell lysate. Our analysis of the experimental data suggests that using DNA–peptide probes can readily facilitate simultaneous analysis of more than 10 miRNAs.



MicroRNA (miRNA) are short, noncoding RNA which are known to regulate gene expression. They bind to the 3′ untranslated regions of target mRNA, and depending on complementarity, either degrade the mRNA or inhibit translation. Deregulation of specific subsets of miRNA, (termed "miRNA fingerprints") has been linked to diseases, such as cancer, giving them potential for use in diagnostics.[1−4] Several studies have shown that these fingerprints only require 2−15 miRNA species to distinguish cancerous from noncancerous tissues,[3,5−8] and to even distinguish specific cancer cell subtypes.[3] Thus, no more than 15 miRNA species would typically be required for a diagnostic miRNA assay. There is a need for a method to detect such fingerprints, which requires direct, quantitative analysis of multiple miRNAs. For use in diagnostics, the method must also be rugged and cost-efficient.

There are many current miRNA detection methods with great sensitivity (qRT-PCR, SPR) and great multiplexing ability (microarrays, next generation sequencing).[9] However, the majority of these methods are indirect, requiring chemical or enzymatic modification of the target miRNA.[10−13] The indirect nature of these methods introduce sequence-specific biases, which reduce quantitative accuracy.[14−16] There has been a wave of direct detection techniques with diagnostic potential: electrochemical-based methods, bioluminescence, two-probe single-molecule fluorescence, isotachophoresis, and capillary electrophoresis (CE)-based methods.[9,17−23] These techniques are all in early stages of development and still require significant efforts to make them practical. Here, we focus on advancing one such method termed direct, quantitative analysis of multiple miRNAs (DQAMmiR).[23]

DQAMmiR is a hybridization assay introduced in 2011 that uses CE with laser-induced fluorescence (CE-LIF) detection to separate and detect multiple miRNAs.[23] An excess of fluorescently labeled, miRNA-specific DNA probes is allowed to bind to target miRNAs. Separation of the free probes from the probe−miRNA hybrids is achieved by the introduction of single-strand DNA binding protein (SSB) into the running buffer. SSB specifically binds to the excess DNA probes, causing a shift in their mobility. For detection of multiple miRNAs, the different probe−miRNA hybrids must be separated, which is difficult due to the similarity in size of all miRNAs. This was overcome by the conjugation of drag tags to the DNA probes, altering their respective electrophoretic mobilities.[24,25] This shift in mobility allowed for the separation of the different

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miRNA–DNA probe hybrids. The peak areas of the excess probe and the individual hybrids could then be used to accurately quantitate the miRNA using a simple algebraic formula. DQAMmiR is a calibration-free approach, allowing it to be robust and rugged enough for diagnostic applications.

In our proof of principle work we detected three miRNAs simultaneously.[23] A detection limit (which is defined as the number of miRNA copies that corresponds to a peak with a signal-to-noise ratio equal to 3) of 3×10^5 copies of miRNA was achieved with a commercial CE-LIF instrument. To explore possible ways of improving the detection limit, we recently combined CE with confocal time-resolved fluorescence (CTRF) detection through an embedded capillary interface (ECI).[26] CE-CTRF-ECI allowed for the detection of 1000 copies (100 pM) of miRNA without modification or enrichment. Practical use of DQAMmiR, e.g., in cancer profiling, will certainly require a better concentration limit of detection, discrimination from both precursor miRNA and similar miRNA sequences, and a capability of analyzing a greater number of miRNA species simultaneously. Lowering the limit of detection will require the use of a preconcentration technique.[20] One-nucleotide specificity of multiple miRNA can be resolved with the introduction of LNA bases into the DNA probes.[27] Precursor miRNA can be analyzed separately by using probes specific to it. The goal of the current work was to find a solution for increasing the number of miRNA species that could be analyzed by DQAMmiR, simultaneously.

We previously used a biotin molecule and a hairpin loop-forming DNA extension as drag tags.[23] Though these two drag tags were able to achieve sufficient hybrid separation, they cannot be easily extended to increase the number of detectable hybrids. Practical applications of DQAMmiR require a more generic extendable drag tag that would allow for an increased number of detectable miRNA. Jiang et al. were able to detect multiple microRNA using drag tags in capillary gel electrophoresis. They attached adenosine tails of varying length to multiple miRNA-specific DNA probes.[28] Adjacent to the miRNA complementary sequence, each DNA probe also contained a DNA sequence complementary to a short, universal, fluorophore-labeled probe. Upon miRNA binding, the universal probe is able to bind via a base-stacking effect. When both the miRNA and universal probe were bound they were ligated together by T4 ligase. They were able to achieve a very good limit of detection (190 fM) and specificity. However, the indirect nature of the ligation may cause sequence-specific biases as previously explained. Furthermore, in gel electrophoresis, the polymerized gel has to be replaced after every run, making this technique quite tedious.

We thought that polymers were a logical choice for an extendable drag tag. Polymers would allow for easy extension, by simply increasing the number of monomers. We decided on using short peptides, which are commercially available, can have a precise number of amino acids (aa), and have been used previously as drag tags in DNA sequencing.[29] We recently published a rigorous theoretical analysis on the use of peptides as drag tags that determined the required number of peptides that would allow sufficient separation of multiple hybrids.[30] We determined that the peptide drag tag lengths must vary between 0 and 20 amino acids to detect five miRNA and between 0 and 47 amino acids to detect nine miRNA.

Using this theoretical work we decided to detect five miRNA using drag tags from 0 to 20 amino acids in length. One untagged probe and four tagged probes were developed by conjugating a neutral five amino acid repeat $(Gly\text{-}Ala\text{-}Gly\text{-}Thr\text{-}Gly)_n$, (where n = 0, 1, 2, 3, 4) to DNA sequences complementary to the five respective miRNA. We measured migration times of tagged hybrids to the detector and found that the migration time decreased with the increased length of the peptide tag. Given the presence of electroosmotic flow (EOF), such behavior of the migration time means that the hybrid electrophoretic mobility decreased with the increased length of the tag as predicted.[30] This conclusion follows from the fact that both untagged and tagged hybrids migrate against EOF that is directed to the detector in our experiments. The five hybrids could be separated from each other, and the tags did not interfere with SSB binding to the excess probes. As a result, we were able to quantitatively detect five miRNA species.

RESULTS AND DISCUSSION

Hybridization Probes. We developed DNA probes with drag tags of 0, 5, 10, 15, and 20 amino acids in length for five miRNAs (mir125b, 155, 21, 10b, and 145, respectively), which are known to be deregulated in breast cancer. Due to the similar length of all five miRNA (mir125b and 21 have 22 nucleotides, mir155, 10b, and 145 have 23 nucleotides) the peptide lengths were randomly chosen for the five probes, regardless of probe length. The DNA and peptides were conjugated with a thioester bond and purified by high-performance liquid chromatography (HPLC). Prior to testing the separative abilities of these drag tags, we had to ensure that the peptides had no hindering effects on our analysis. The peptides could potentially interfere with miRNA binding to its respective probe, causing either a reduction in quantitative accuracy or slowing binding, thereby increasing hybridization time. We also had to determine whether the drag tags could hinder SSB binding to the DNA, which is required for all DNA probes in DQAMmiR. Due to the short lengths of peptides used, we did not expect any negative effects.

SSB binds nonspecifically to all DNA probes, and due to its large size, causes all probes (regardless of drag tag size) to migrate with the same mobility, forming a single peak. Shifting all probes to a single peak allows us to have a large separation window, which we utilized for detecting multiple hybrid peaks. If peptide drag tags interfered with SSB binding, the separation window would be significantly reduced, preventing the detection of multiple miRNA. To observe the impact of the drag tags on SSB binding we injected all five DNA–peptide probes into the capillary with SSB in the run buffer (Figure 1). All five probes bound to the SSB and formed a single peak, showing that the drag tags did not interfere with SSB binding.

To ensure that the peptide drag tags did not interfere with the probes ability to bind to miRNA, we incubated each of the five miRNA species with their respective DNA probe, both with and without the drag tag. In agreement with our previous work,[23] the drag tags did not interfere with miRNA binding (data not shown). Thus, the new peptide drag tags did not interfere with either SSB or miRNA binding, allowing for their use in DQAMmiR.

Separation of Hybridization Probes. We next studied the mobility of the five DNA–peptide probes. Though the theoretical work was based on the separation of hybrids, we assumed that a similar shift would be observed when the five probes were injected into the capillary without either miRNA or SSB present. Though all of the drag tags caused a mobility shift relative to the untagged probe (Figure 1), there was an unexpected lack of separation between the 15 and 20 amino

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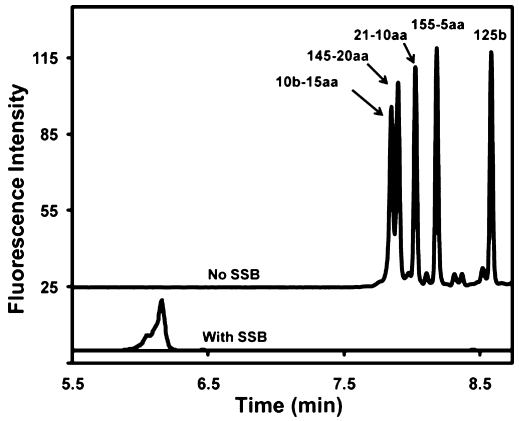

Figure 1. Separation of the five DNA probes. Upper: peptides drag tags with lengths of 0, 5, 10, 15, and 20 amino acids were conjugated to DNA probes for mir125b, 155, 21, 10b, and 145, respectively. The peptides caused a mobility shift from the untagged probe (125b). The DNA probes 10b−15aa and 145−20aa have a similar mobility. Lower: presence of SSB in running buffer caused all five probes to shift into a single peak.

acid drag tag probes. Interestingly, the DNA probe with 15aa had a more significant mobility shift than the 20aa probe. The mobility of single-stranded DNA was not the focus of this paper, so we did not explore why this occurred; however, we speculate that it was most likely due to the DNA probes having dissimilar conformations when not in double-stranded form.

Separation of miRNA−Probe Hybrids. Regardless of the lack of separation between the 15aa and 20aa probes we went on to test the separation between the five hybrids. We incubated all five DNA probes with their respective miRNA and injected a small plug into the capillary (Figure 2). We observed

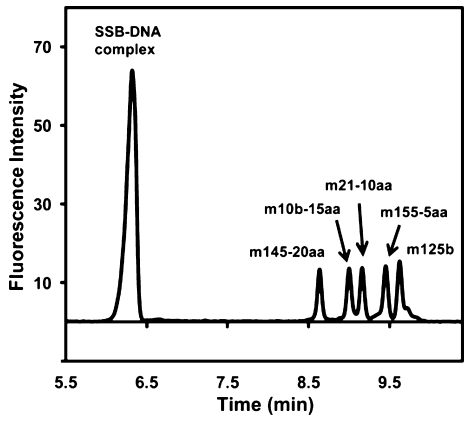

Figure 2. Separation of five miRNA-probe hybrids: 50 nM of the five DNA probes (125b, 155−5aa, 21−10aa, 10b−15aa, 145−20aa) and 10nM of their respective miRNA were injected into the capillary with 50 nM SSB in the running buffer. Five distinct hybrid peaks are present, with sufficient separation between hybrids to allow quantitation. Peak areas are normalized by quantum yields.

five distinct peaks representing each of the respective hybrids. The lack of separation that occurred with the ssDNA probes was resolved, most likely due to the conformational rigidness of a double-stranded hybrid. The separation between each of the hybrids was not uniform with the mir125b and mir155, as well as the mir21 and mir10b peaks, being relatively close. This was due to the slight difference in miRNA length as previously mentioned. The 22 nucleotide long hybrids (mir125b, mir21) have a lower electrophoretic mobility than the longer 23 nucleotide hybrids (mir145, mir10b, mir155) regardless of drag tag, which is in agreement with our theoretical work.[30] If required, greater separation could easily be achieved by changing the drag tag and probe combinations; however, we wanted to show that, regardless of miRNA length, separation could be achieved. Though the five miRNA detected here are of similar length, miRNA of varying length can also be simultaneously detected. As long as the shorter DNA probes are conjugated to the longer peptides, sufficient separation between hybrid peaks will be achieved.[30]

We compared the experimental migration time (t_{det}) of the peaks shown in Figure 2 with theoretically predicted ones. It should be noted that all theoretical work was calculated using the same conditions (buffer composition, pH, ionic strength, etc.) present in our experimental work. To calculate t_{det} one should take into account the following relations:[30]

$$u = u_{EOF} + \mu_{hyb+tag}E, \quad \mu_{hyb+tag} = \frac{\mu_{hyb}}{1 + \frac{6\lambda_D R_{H,tag}}{d_{hyb}L_{hyb}}} \tag{1}$$

Here, u_{EOF} and u are velocities of EOF and hybrids (untagged or tagged), E is the electric field strength, μ_{hyb} and $\mu_{hyb+tag}$ are the mobilities of untagged and tagged hybrids, L_{hyb} and d_{hyb} are the length and diameter of hybrids, λ_D is the Debye length of the buffer, and $R_{H,tag}$ is the hydrodynamic radius of the tag ($R_{H,tag}$ = 0 for untagged hybrids). The mobilities of untagged and tagged hybrids are negative since the hybrid is negatively charged. A value of $R_{H,tag}$ is related to the gyration radius $R_{G,tag}$ of tag by the relation

$$\rho R_{H,tag} = R_{G,tag} \tag{2}$$

where $R_{G,tag}$ is determined by the Kratky−Porod equation and ρ = 1.5 for a Gaussian coil.[31,32] Given an increase in electric field strength (from E_0 = 100 V/cm to E = 500 V/cm) after the initial period of time t_0 = 3 min, t_{det} is calculated as follows:

$$t_{det} = \frac{L_{det} - (u_{EOF,0} + \mu_{hyb+tag}E_0)t_0}{u_{EOF} + \mu_{hyb+tag}E} + t_0 = \frac{L_{det}}{u_{EOF} + \mu_{hyb+tag}E} + t_0\left(1 - \frac{E_0}{E}\right) \tag{3}$$

Here, L_{det} is the distance to the detector and $u_{EOF,0}$ is the velocity of the initial EOF corresponding to E_0 = 100 V/cm. In relation 3 we also used proportionality between the EOF velocity and the electric field strength. In deriving relations 1−3 we assumed that (i) a miRNA−DNA hybrid behaves like a rigid rod moving parallel to its axis, (ii) the hybrid diameter is higher than the Debye length λ_D of the buffer, and the hybrid length is many times higher than λ_D, (iii) the electric charge is uniformly distributed along the hybrid length, (iv) Manning's counterion condensation results in the hybrid charge normalization, (v) drag tags are uncharged, and (vi) fluid resistance of a drag tag can be described by its hydrodynamic radius related to the gyration radius by relation 2.

In Figure 3, we show a comparison of the experimentally observed migration times with their theoretical estimates based on relations 1−3. The red solid line shows theoretical results for ρ = 1.5 and $\mu_{hyb} = -3.1 \times 10^{-4}$ cm^2/sV (Figure 3). Clearly, they do not perfectly match our experimental results (black circles). The experimental data shows less resolution than what

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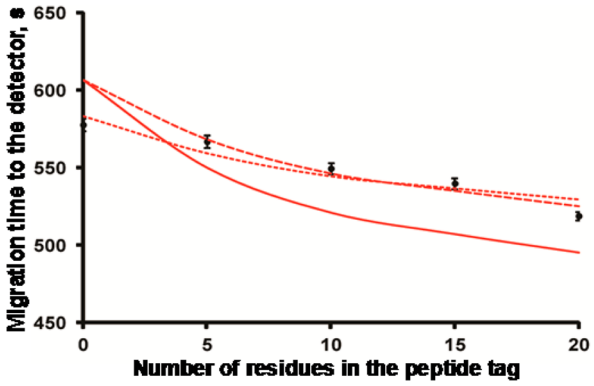

Figure 3. Experimental (black circles with error bars) with error bars and theoretical (red lines) values of migration time to the detector for hybrids with various drag tags. The red solid line corresponds to ρ = 1.5 and $\mu_{hyb} = -3.1 \times 10^{-4}$ cm^2/sV in relations 1 and 2. The red long-dashed line is the best fit of theoretical values that is achieved at ρ = 2.4 when μ_{hyb} is fixed. The red short-dashed line shows the best fit when both ρ and μ_{hyb} are varied. It is achieved at ρ = 3.5 and $\mu_{hyb} = -3.0 \times 10^{-4}$ cm^2/sV.

was theoretically predicted; however, we can still detect all five peaks. Furthermore, the closeness of the peaks in Figure 2 is actually beneficial for allowing a greater number of miRNA to be detected in the limited detection window. Agreement with the theory can be significantly improved if we use a coefficient ρ in relation 2 as an adjustable parameter. The red long-dashed line shows theoretical migration times for ρ = 2.4. This value corresponds to the best fit of theoretical results to experimental ones when μ_{hyb} is fixed. The best fit was found by calculating normalized root mean square deviation (NRMSD) between experimental and theoretical values of t_{det} at various values of ρ.[33] We have NRMSD = 4.6% and 2.3% before and after the ρ adjustment, respectively. It turned out that varying both ρ and μ_{hyb} in the fitting procedure leads to ρ = 3.5, $\mu_{hyb} = -3.0 \times 10^{-4}$ cm^2/sV, and NRMSD = 1.2%. Corresponding theoretical results are depicted by the red short-dashed line in Figure 3. The value of μ_{hyb} obtained from the best fit is fairly close to the value $\mu_{hyb} = -3.1 \times 10^{-4}$ cm^2/sV that was theoretically calculated for miRNA hybrids and corresponds to observed DNA mobility.[30,32] Thus, our initial theory correctly predicts the mobility of untagged hybrids.[30] Values of ρ found from the best fit show that peptide drag tags do not behave exactly as a Gaussian coil, which is not surprising, given their relative shortness. Indeed, for the rodlike drag tag oriented along its velocity we would have[30,31]

$$R_H = \frac{nl}{3}\left(\ln\frac{2nl}{d} - \frac{1}{2}\right)^{-1}, \quad R_G = \frac{nl}{\sqrt{12}} \tag{4}$$

where n is the number of residues in the drag tag and l and d are the length and diameter of one residue. Relations 4 lead to ρ = 2.5 and 3.1 at n = 10 and 20, respectively, where we assume that l = 0.36 nm and d = 0.24 nm.[30] Thus, values of ρ obtained from the best fit suggest that the drag tag conformation looks more like a rod rather than a Gaussian coil.

Given that the experimental and theoretical peak migration times reasonably agree, we proved that, in agreement with our initial theory, drag tags ranging from 0 to 20 amino acids were sufficient for the detection of five miRNA. This allows us to confidently design DNA probes with sufficient drag tag lengths to detect an even greater number of miRNA. The quality of separation of the hybrids from each other and the hybrids from the SSB-bound excess probes suggests that up to 25 miRNA can be analyzed simultaneously by further increasing drag tag lengths.

Quantitation. Though the peptide drag tags did not interfere with miRNA binding, they did have a slight quenching effect on the probe signal. The quenching varied between the probes. We were able to take this quenching effect into account to maintain quantitative accuracy. We also determined the quantum yields of each of the DNA probes with respect to when they are SSB bound and miRNA bound. The following formula was used to determine the concentrations of the ith miRNA using the SSB−DNA and miRNA−DNA hybrid peaks from their respective electropherograms (derivation of the equation can be found in our previous work):[23]

$$[\text{miRNA}]^i = \frac{A_H{}^i \sum_i^N [P]_0{}^i q_P{}^i}{q_H{}^i[(\sum_i^N A_H{}^i q_P{}^i/q_H{}^i) + A_P]} \tag{5}$$

where $[P]_0{}^i$ is the total concentration of the ith probe (composed of the hybrid and the miRNA-unbound probe), A_H is the area corresponding to the ith hybrid, A_P is the cumulative area of the excess probe, $q_H{}^i$ is the relative quantum yield of the ith hybrid with respect to that of the free probe, and $q_P{}^i$ is the relative quantum yield of the ith probe in the presence of SSB with respect to that of the free probe. Using this formula we were able to accurately determine the concentrations of all five miRNA, ranging from 100 pM to 100 nM (Figure 4).

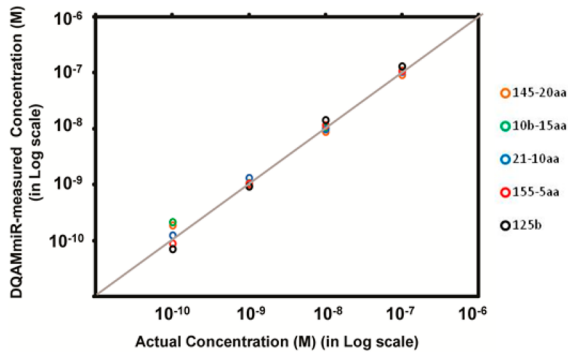

Figure 4. Quantitative analysis of five miRNA was processed simultaneously using eq 5. Excess (500 nM) of the five DNA probes was incubated with all five respective miRNA, ranging in concentration from 100 pM to 100 nM. The different concentrations of miRNA were prepared by serial dilution of a stock solution. The concentration of the stock solution was determined by light absorbance at 260 nm.

Detection of Spiked miRNA in MCF-7 Cell Lysate. For proof of principle, the new probes were introduced into a complex biological matrix to show their potential in biological samples. The five DNA probes were incubated with MCF-7 cell lysate spiked with miRNA (Figure 5). Masking DNA and RNA were included in the sample to prevent probe and miRNA degradation, respectively. Though the peak height of the mir125b hybrid appears to be greater than the other peak heights, the peak areas remain similar (Supporting Information) showing that the lysate had negligible effect on the quantitative analysis of all five hybrid peaks (Figure 5). Since we could not detect all five endogenous miRNA targets directly from the cell lysate, we spiked in miRNA at a concentration

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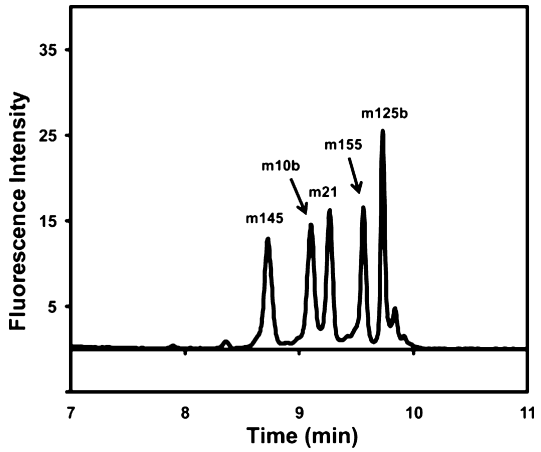

Figure 5. Detection of miRNA from a biological matrix: 50 nM of the five DNA probes (145−20aa, 10b−15aa, 21−10aa, 155−5aa, 125b) and 10nM of their respective miRNA were spiked into MCF-7 cell lysate. All five hybrid peaks were observed. Small peaks to the left of the miRNA peaks indicate impurities from the lysate.

greater than biologically relevant (picomolar or lower) concentrations.

MATERIALS AND METHODS

Materials. All DNA and miRNA for hybridization assays were custom synthesized by IDT (Coralville, IA, U.S.A.). The miRNA and DNA probe sequences can be found in the next section. All maleimide-modified peptides were synthesized by Canpeptide (Pointe-Clare, QUE, Canada). *E. coli* single-strand DNA binding protein (SSB) was purchased from Epicenter Biotechnologies (Madison, WI, U.S.A.). All other materials were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

Oligonucleotides and Peptides. The five miRNAs had the following sequences: mir10b, 5′-UACCCUGUAGAACCGAAUUUGUG-3′; mir21, 5′-UAGCUUAUCAGACUGAUGUUGA-3′; mir125b, 5′-CCUGAGACCCUAACUUGUGA-3′; mir145, 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′; mir155, 5′-UUAAUGCUAAUCGUGAUAGGGGU-3′.

The hybridization probes had the following sequences: probe for mir10b, 5′-thiolC6S-S-CACAAATTCGGTTCTACAGGGTA-Alexa488-3′; probe for mir21, 5′-thiolC6S-S-TCAACATCAGTCTGATAAGCTA-Alexa488-3′; probe for mir125b, 5′-thiolC6S-S-TCACAAGTTAGGGTCTCAGGGA-Alexa488-3′; probe for mir145, 5′-thiolC6S-S-AGGGATTCCTGGGAAAACTGGAC-Alexa488-3′; probe for mir155, 5′-thiolC6S-S-ACCCCTATCACGATTAGCATTAA-Alexa488-3′.

All of the peptides contained a maleimide modification on their N-terminus. The following amino acid sequences were used with respect to the DNA probe they were conjugated with: probe for mir155, C-term-Gly-Ala-Gly-Thr-Gly-N term; probe for mir21, C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term; probe for mir10b, C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term; probe for mir145, C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term.

Conjugation of DNA with Peptides. All DNA probes contained a disulfide modification on their 5′ end which needed to be reduced prior to conjugation. Tris(2-carboxyethyl)-phosphine (TCEP) was used as the reducing agent. An amount of 300 μL of TCEP reducing beads (Fisher Scientific, Ottawa, ON, Canada) was spun down at 10 000 rpm, and the supernatant was removed. The beads were washed with a modified PBS containing 500 mM NaCl at pH 7.0 and spun again at 10 000 rpm. The PBS wash was removed, and 2 μM of the DNA probe was incubated with the TCEP at 37 °C with vigorous shaking for 2 h. The sample was spun down at 10 000 rpm for 5 min, and the filtrate, containing the reduced DNA, was added to an equal volume of 20 μM maleimide-modified peptide. The final concentrations of the DNA and peptide were 1 and 10 μM, respectively. The conjugation reaction was carried out for 1 h at 37 °C with vigorous shaking. Purification was performed immediately after conjugation.

Purification of DNA−Peptide Conjugate. The DNA−peptide conjugate was purified from the excess of non-conjugated DNA by HPLC. HPLC was performed on a System Gold system (Beckman Coulter, Fullerton, CA, U.S.A.) equipped with an FP-2020 Plus fluorescence detector (Jasco, Easton, MD, U.S.A.) and Gen-Pak FAX anion-exchange column (Waters, Milford, MA, U.S.A.). The eluted conjugate was collected, and the HPLC buffer was exchanged with deionized H_2O by centrifuging with an Amicon Ultracel 3K filter (Millipore, Billerica, MA). The sample was frozen at −80 °C and lyophilized for storage.

Hybridization Conditions. Hybridization was carried out in Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Various concentrations of the miRNA species (mir10b, 21, 125b, 145, 155) were incubated with 500 nM of their respective hybridization probes along with 50 nM bodipy (internal standard) in the incubation buffer (50 mM Tris−Ac, 50 mM NaCl, 10 mM EDTA, pH 8.2). 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 for 1 h to allow annealing. To minimize miRNA degradation, a nuclease-free environment was used while handling miRNA samples.

CE-LIF. All experiments were performed using a P/ACE MDQ CE instrument (Beckman-Coulter, Fullerton, CA, U.S.A.) equipped with an LIF detector. 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 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 100 V/cm for 3 min, followed by an electric field of 500 V/cm for 15 min with normal polarity and coolant-controlled temperature maintained at 20 °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 internal standard, bodipy. Concentrations of miRNA were determined using eq 5.

DQAMmiR in Cell Lysate. MCF-7 cells were purchased from ATCC and grown in an incubator at 37 °C in the atmosphere of 5% CO_2. Cells were grown in DMEM media (Invitrogen) with FBS and 10 000 μg/mL penicillin, streptomycin in a 100 mm Petri dish. When cells covered roughly 90% of the plate they were washed with PBS, trypsinized to detach them from bottom of dish, and

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centrifuged at 300*g* for 5 min. Pellet was washed twice with PBS. The cells were counted using a hemocytometer and lysed with 1% Triton in the incubation buffer with 10 μM masking RNA (tRNA library from baker's yeast). Cell lysates were aliquoted and stored at −80 °C. A 10 times diluted cell lysate was incubated with 50 nM of each of the five DNA probes, 10 nM of each miRNA (mir10b, 21, 125b, 145, 155), 50 nM bodipy, and 1 μM masking DNA. Masking DNA was a 20-nt DNA strand, with the sequence of 5′-CAAAAAATGAGTCAT-CCGGA-3′. Hybridization, injection, and capillary conditions were performed as previously explained.

CONCLUSIONS

Here we have demonstrated the design of DNA probes with a universal drag tag and in a proof-of-principle DQAMmiR experiment achieved separation of five hybrids. The use of a universal drag tag allows for an increasing number of miRNA to be detected by simply increasing the number of amino acids. The agreement between our experimental and theoretical analysis allows for future DNA probes to be designed with confidence that the respective hybrids will achieve baseline separation allowing for quantitation. Our experimental results suggest that we can separate up to 25 hybrids. Universal drag tags simplify DNA probe design, increasing the number of detectable miRNA. This allows for DQAMmiR to be used in a wider array of applications. Future work should focus on further improving the limit of detection to allow for the analysis of low expression level miRNA species from biological samples.

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.

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SUPPORTING INFORMATION

Universal Drag Tag for Direct Quantitative Analysis of Multiple microRNAs (DQAMmiR)

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Tables of quantum yield values and DQAMmiR-measured miRNA concentrations

Quantum yields of the DNA probes for the respective miRNA. q_P is the quantum yield of SSB-bound probe and q_H is the quantum yield for the DNA probe-miRNA hybrid. These values were determined as explained in our previous work [24].

Quantum Yield	Mir145-20aa	Mir10b-15aa	Mir21-10aa	Mir-155-5aa	Mir125b
q_P	0.13 ± 0.01	0.14 ± 0.01	0.22 ± 0.01	0.17 ± 0.03	0.24 ± 0.02
q_H	0.36 ± 0.09	0.58 ± 0.07	0.68 ± 0.09	0.63 ± 0.16	0.63 ± 0.08

After the conjugation of peptides to the DNA, the variation of fluorescence intensity had to be taken into account. The fluorescence intensity of all DNA probes was normalized by determining their quantum yields with respect to the untagged DNA probe (q_D).

Quantum Yield	Mir145	Mir10b	Mir21	Mir-155	Mir125b
q_D	0.38 ± 0.01	0.58 ± 0.01	0.46 ± 0.003	0.47 ± 0.003	1

The quantum yields with respect to miRNA-binding and SSB-binding can simply be adjusted by multiplying by q_D

Adjusted Quantum Yield	Mir145	Mir10b	Mir21	Mir-155	Mir125b
q_P	0.05 ± 0.004	0.08 ± 0.006	0.11 ± 0.006	0.08 ± 0.014	0.24 ± 0.02
q_H	0.14 ± 0.035	0.34 ± 0.042	0.31 ± 0.043	0.30 ± 0.076	0.63 ± 0.08

Quantitative analysis of 10nM target miRNA species spiked into either MCF-7 cell lysate or lysate-free buffer

	DQAMmiR-calculated miRNA concentration (nM)				
Sample	**Mir145**	**Mir10b**	**Mir21**	**Mir-155**	**Mir125b**
MCF-7 cell lysate	9.04 ± 0.068	10.72 ± 0.36	10.63 ± 0.33	9.77 ± 0.66	11.82 ± 0.89
Lysate-free buffer	8.81 ± 0.58	10.37 ± 0.39	9.78 ± 0.57	11.52 ± 0.50	12.01 ± 0.36