Direct Quantitative Analysis of Multiple miRNAs (DQAMmiR)**

David W. Wegman and Sergey N. Krylov[*]

MicroRNAs (miRNAs) are short RNA molecules (18-25 nucleotides) that were recently proven to play an important role in regulation of cellular processes,[1,2] and their abnormal expression is associated with pathologies such as cancer.[3,4] The change in the cellular status is typically associated with simultaneous change in the level of several miRNAs.[5-11] For example, abnormal expression of 2 miRNAs was found to be indicative of colorectal cancer in humans.[12] Therefore, both studying the biological role of miRNA and using miRNA for informative disease diagnostics require *accurate quantitative* analyses of *multiple* miRNAs. Most methods of miRNA detection are indirect (e.g. PCR, microarrays, SPR, next generation sequencing, etc.) – they require chemical or enzymatic modifications of miRNA prior to the analysis.[13-16] Not only do the modifications make the analysis more complex and time-consuming but they also reduce its accuracy due to different efficiencies of modifications for different miRNAs.[17-19] There are a few direct methods, which do not require any modification of the target miRNA. Northern blotting does not require any modifications; however, the methods can be tedious and though it can be

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quantitative analysis of multiple miRNAs. Here we report the first direct quantitative analysis of multiple miRNAs (DQAMmiR). DQAMmiR uses miRNAs directly, without any modification, and accurately determines concentrations of multiple miRNAs without the need to build calibration curves. This was achieved by capillary electrophoresis-based hybridization assay with an ideologically-simple combination of two well-known separation-enhancement approaches: (i) drag tags on the DNA probes,[26,27] and (ii) single-strand DNA binding protein (SSB) in the run buffer.[28] In this proof-of-principle work, we developed DQAMmiR for 3 miRNAs (mir21, 125b, 145) known to be deregulated in breast cancer. DQAMmiR opens the opportunity for simple, fast, and quantitative fingerprinting of up to several tens of miRNAs in basic research and clinical applications. The availability of suitable for DQAMmiR commercial instruments makes the method practical for a large community of researchers.

We based DQAMmiR upon a classical hybridization approach in which hybridization probes, labeled for detection and taken in excess to miRNAs, bind miRNAs sequence-specifically. Electrophoresis can be used to efficiently separate oligonucleotides,[29] but simultaneously separating the hybrids from each other and from the unbound probes is challenging and so far has not been achieved.[30] We solved the separation problem through a combination of two well-known mobility-shift approaches: (i) drag tags on the probes[26] and single strand DNA binding (SSB) protein in the run buffer.[28] **Figure 1** illustrates this hypothetical approach where the miRNAs and their complimentary ssDNA probes are shown as short lines of the same color, drag tags are shown as parachutes, a fluorescent label is shown as small green circles and SSB is shown as large black circles. In the hybridization step, the excess of the probes is mixed with the miRNAs which leads to all
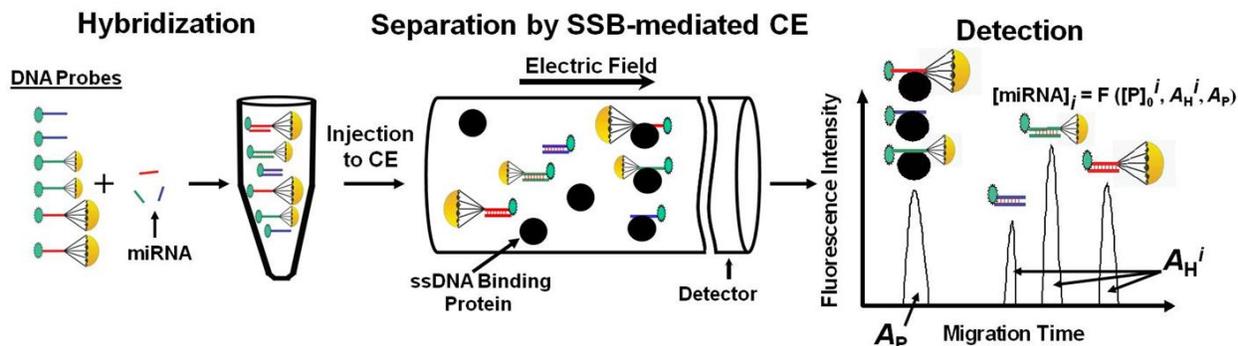

Figure 1. Schematic representation of the direct quantitaive analysis of miRNA. See text for details.

quantitative it's sensitivity is limited. Signal amplifying ribozymes, *in situ* hybridization, bioluminescence detection, and two-probe single-molecule fluorescence are other direct miRNA detection methods[20-24] however, the first two are only semi-quantitative while the latter two can hardly be used for multiple miRNAs. Cheng *et al.* used rolling-circle amplification (RCA) which does not require modification of the miRNA to detect low concentrations of miRNA and could be run in parallel; however, the process is tedious taking 8 hours and the amplification step can potentially lead to biases quantiation.[25] Thus, there is currently no method for direct

miRNAs' being hybridized but with some probes left unbound to miRNA. A short plug of the hybridization mixture is introduced into a capillary prefilled with an SSB-containing run buffer. SSB binds all ssDNA probes but does not bind the double stranded miRNA-DNA hybrid. When an electric field is applied, all SSB-bound probes move faster than all the hybrids (SSB works as a propellant).[28] Different drag tags make different hybrids move with different velocities. SSB-bound probes, however, can move even with similar velocities if the drag tags are small with respect to SSB. In such a case, a fluorescent detector at the end of the capillary
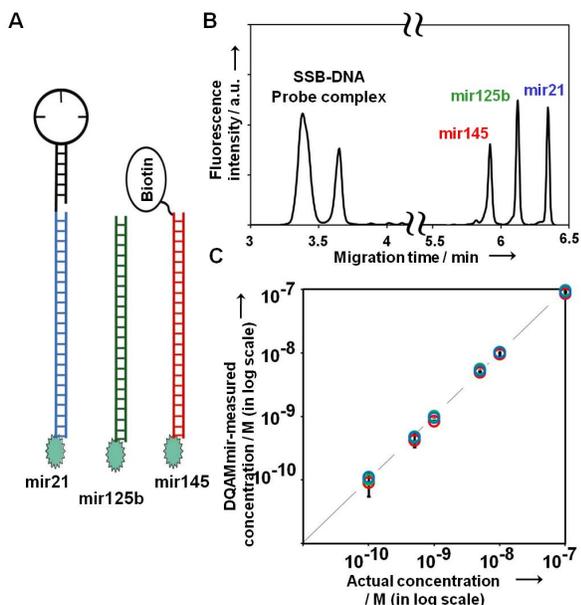
Figure 2. **a)** Structures of 3 miRNA-DNA probe hybrids. **b)** CE separation of the 3 hybrids from each other and from the excess probe facilitated by the drag tags on mir21 and mir145 and SSB in the run buffer. The concentrations of miRNAs were 5 nM each and the concentration of the probes were 50 nM each. **c)** Quantitative properties of the analysis utilizing data of panel B at concentrations of miRNAs varying from 100 pM to 100 nM (three repetitions) processed with **Eq. 1**. Different concentrations of miRNA were prepared by serial dilution of a stock solution. The concentration of the stock solution was determined by light absorbace at 260 nm. Standard deviation of DQAMmiR-measured miRNA concentrations was 6.6%.

generates separate signals for the hybrids and a cumulative signal (one peak or multiple peaks) for the excess of the probes. The amounts of miRNAs are finally determined with a simple mathematical approach that uses the integrated signals (peak areas in the graph). We reserve the term of direct quantitative analysis of multiple miRNAs and its abbreviation of DQAMmiR for the specific approach described above.

To experimentally test the viability of our hypothetical DQAMmiR, we decided to use three miRNAs known to be deregulated in breast cancer: mir21 (5'-UAGCUUAUCAGA CUGAUGUUGA-3'), mir125b (5'-UCCCUGAGACCCUAACUU GUGA-3'), and mir145 (5'-GUCCAGUUUUCCCAGGAAUCCC U-3'). Three ssDNA probes were designed all labeled with Alexa 488 at the 5' end; the 3' end was reserved for drag tags. To separate the three hybrids we needed only two probes modified with drag tags; one probe could be without a drag tag. In the proof-of-principle work, we chose to use the two simplest available drag tags: a hairpin formed by a DNA extension at the 3' end of the probe and biotin covalently attached to the 3' end. The probe for mir21 was the one with the hairpin formed by the italicized extension: 5'-Alexa488TCAACATCAGTCTGATAAGCTA*GCGCGCTTTGCGC GC*-3'. The probe for mir125b was the one with no tag: 5'-Alexa 488-TCACAAGTTAGGGTCTCAGGGA-3'. The probe for mir145 was the one with biotin: 5'-Alexa 488-AGGGATTCCTGGGAAAACTGGAC-Biotin-3'.

The experimental details are described in the supporting information. Briefly, the miRNAs were hybridized with the probes in the incubation buffer (50 mM Tris-Ac, 50 mM NaCl, 10 mM EDTA, pH 7.8) containing fluorescein as an internal standard by first increasing temperature to a denaturing 80°C, then lowering it to 37°C at a rate of 20°C/min and finally keeping it at 37°C for one

hour to allow complete hybridization. To minimize miRNA degradation, a nuclease-free environment was used while handling miRNA samples. The structures of the three hybrids formed are schematically depicted in **Fig. 2A**. The separation was carried out in a bare fused silica capillary with a positive electrode at the injection end of the capillary. The capillary was pre-filled with the SSB-containing run buffer: 25 mM sodium tetraborate at pH 9.3 supplemented with 50 nM SSB. Under such conditions, an electroosmotic flow occurred and moved negatively charged hybrids and probes to the detection end of the capillary where the negative electrode was situated. A commercial CE instrument, with fluorescence detection suitable for the Alexa488 label on our probes, was used. Samples were injected 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 a capillary coolant temperature set at 20ºC.

Figure 2B shows the result of electrophoretic separation in DQAMmiR which agrees with a hypothesis depicted in **Fig. 1**. SSB bound the excess probes and increased their mobility generating two adjoining peaks at approximately 3.4 and 3.6 min. The hybrids had no ssDNA parts accessible for SSB to bind and, therefore, SSB did not affect their mobility. The negatively charged hairpin slowed down the mir21 hybrid, while neutral biotin increased the velocity of the mir145 hybrid with respect to the mir125b hybrid. All hybrid peaks were perfectly resolved and their areas could be accurately determined, which, in turn, allowed us to determine the quantities of the three miRNAs. The time window between the SSB-bound probes and the hybrids was 2 min. With the observed peak widths of the hybrids, the 2-min window is sufficient to resolve a maximum of approximately 20 peaks. While this maximum can be increased by optimizing the separation conditions, it is unlikely to exceed 30-40. This is the electrophoresis-associated limit for the maximum number of miRNAs that can be analyzed by DQAMmiR with fluorescence detection in a single spectral channel.

One of the major requirements of miRNA analyses is selectivity; any miRNA detection method should be able to discriminate miRNA from a similar sequence with a single nucleotide being different. Such selectivity is typically based on the difference in melting temperatures between the full-match and single-nucleotide-mismatch hybrids. We studied the selectivity of a single DNA probe and confirmed that by increasing the temperature of the electrolyte above the melting temperature of the single-nucleotide-mismatch hybrid but below the melting temperature of the perfect-match hybrid completely eliminated the peak from the mismatch while not affecting the peak of the match. Moreover, due to the thermal stability of SSB, our concept worked equally well at the elevated temperature (see Supporting Information). Thus, DQAMmiR has the potential for a single-nucleotide sensitivity required for miRNA detection in biological samples.

To determine the quantities of individual miRNA from the experimental data similar to that in **Fig. 2B**, we developed the mathematics of DQAMmiR that does not require resolving SSB-bound probes and takes into account a potential change of the quantum yield of fluorescence of the probe upon its binding to miRNA or SSB. The derivations can be found in the supporting information. Here we present the resulting equation for the calculation of the concentration of the i-th miRNA in the hybridization mixture containing N miRNAs:

$$[\text{miRNA}]^i = \frac{A_\text{H}^{\ i} \sum_{i}^{N} [\text{P}]_0^{\ i} q_\text{P}^{\ i}}{q_\text{H}^{\ i} \left[\left(\sum_{i}^{N} A_\text{H}^{\ i} q_\text{P}^{\ i} / q_\text{H}^{\ i} \right) + A_\text{P} \right]} \quad (1)$$

$[\text{P}]_0^{\ i}$ is the total concentration of the i-th probe (composed of the hybrid and the miRNA-unbound probe), A_H is the area corresponding to the i-th hybrid, A_P is the cumulative area of the
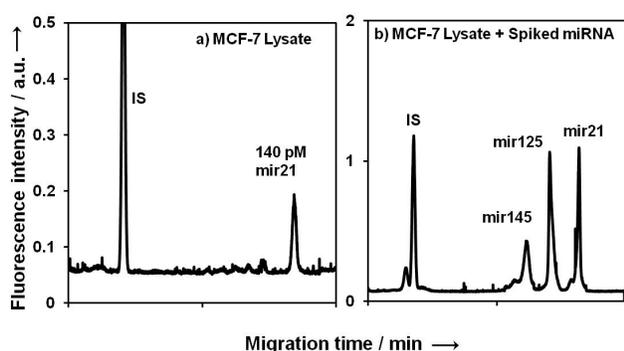
Figure 3. The influence of complex biological matrix on miRNA analysis by DQAMmiR. **a)** DQAMmiR of 3 DNA probes at 5 nM each incubated with MCF-7 cell lysate and masking DNA/RNA in incubation buffer. **b)** DQAMmiR of 3 DNA probes at 5nM each plus 0.5 nM spiked in mir21, mir125b and mir145 incubated with MCF-7 cell lysate and masking DNA/RNA. IS labels the peak of the internal standard (fluorescein).

excess probe, $q_H{}^i$ is the relative quantum yield of the *i*-th hybrid with respect to that of the free probe, and $q_P{}^i$ is the relative quantum yield of the *i*-th probe in the presence of SSB with respect to that of the free probe. To allow a universal equation that can be used when additional DNA probes are introduced to detect an even greater number of miRNA, the peak areas of all SSB-bound probes were combined to form A_P. Refer to the right part of **Fig. 1** for better understanding of the area assignments.

Equation (1) was used to determine the amounts of miRNA in the experiment shown in **Fig. 2B** (the quantum yields were determined in separate experiments described in the supporting information). The results of the calculations shown in **Fig. 2C** demonstrate the high accuracy (94%) and great signal linearity (R = 0.9999) of the DQAMmiR method in the range of at least 3 orders of magnitude. It is important to emphasize that DQAMmiR does not require calibration curves.

After proving the concept of DQAMmiR, we tested the method for its tolerance to a complex biological matrix. The sample was made of the 3 miRNAs added to the *E. coli* cell lysate supplemented with fluorescein as an internal standard (to ensure controlled injection of the relatively viscous crude cell lysate) and masking RNA and DNA (to prevent degradation of miRNA and DNA probes). The hybridization mixture was prepared, processed, and analyzed in the way described above for pure solutions of miRNA. **Figure S2** in supporting information compares the results of DQAMmiR for the cell lysate and for a pure buffer as the sample matrices. Qualitative comparison of the data shows only insignificant differences. Moreover, calculations of miRNA concentrations (**Table S3** in the Supporting Information) also produce similar results, thus confirming that neither the cell lysate nor masking DNA/RNA significantly affected the results and that DQAMmiR could be potentially directly used for complex biological samples without RNA extraction or other sample processing.

To test this, we used DQAMmir on a MCF-7 cell lysate sample which is known to up-regulate mir21 and down-regulate mir125b and mir145. **Figure 3** compares the DQAMmiR results for the pure MCF-7 cell lysate and the lysate with 3 miRNAs spiked in it. In the lysate-only sample a peak for the up-regulated mir21 was detected and the concentration of mir21 was determined to be 140 pM. The correctness of this value was confirmed by analyzing 140 pM mir21 in a pure buffer and observing an identical peak. The peaks of down-regulated mir125b and 145 were below the background noise. This result indicates that available commercial CE instrumentation may not be sensitive enough for DQAMmir of downregulated

miRNAs without their pre-concentration. The ultimate solution of this limitation will be the commercialization of instrumentation with single-molecule fluorescence detection, which exists in experimental prototypes.

Below we outline the major features of DQAMmiR and further directions of its development and applications. The following major parameters are used to characterize any method of miRNA detection: analysis time, number of miRNAs analyzed simultaneously, specificity, amount of sample required, limit of detection, dynamic range, accuracy, and tolerance to biological matrices. With no sample processing involved, the analysis time for DQAMmiR is limited by hybridization and separation times only; it is approximately 1.5 h. The hybridization time can be further shortened by increasing the concentrations of the probes. The resolution between the SSB-bound probes and the hybrids in DQAMmiR (R_s = 23.4) roughly suggests that a maximum of approximately 20 miRNAs can be reliably analyzed in a single spectral channel without further optimization of the analysis. This number can be doubled through using 2 different fluorescent labels and a commercially-available instrument with 2 spectral channels. Design of suitable drag tags and methods of their conjugation to the probes will be crucial for DQAMmiR to reach its electrophoresis limit of the maximum number of miRNA that can be simultaneously analyzed. (With a maximum number of miRNAs analyzed below 50, DQAMmiR cannot compete with microarrays in wide miRNA screens, but is suitable for the majority of other applications). This design may take some time and effort but when the tags are developed and validated no further optimization of them will be required. DQAMmiR is capable of 1-nucleotide differentiation (see Supporting Information), simply by increasing the capillary temperature above the melting temperatures of all the mismatched hybrids. The temperature should be adjusted to keep the full-match hybrids intact. One analysis consumes a fraction of µL of the sample. The limit of detection of DQAMmiR is restricted by that of CE with fluorescence detection. Commercially available CE instruments have a limit of detection of approximately 10^4-10^5 copies of the target molecule for hybridization assays.[31] Custom-designed detectors can have a limit of detection down to hundred of copies.[32] These limits of detection should be sufficient for the majority of biologically relevant assays.[33] The dynamic range of DQAMmiR is limited by the dynamic range of linear response of a fluorescent detector which we found to be at least 3 (between 10^{-10} and 10^{-7} we have 3 rather than 4 orders of magnitude) orders of magnitude. Our proof-of-principle results demonstrate that the method has the accuracy of approximately 94% and precision of approximately 92%. Our experiment with the cell lysate suggests that DQAMmiR is also highly tolerant to impurities in the sample, which makes the method applicable to crude biological samples.

To conclude, DQAMmiR is the first approach that requires no miRNA modification in the sample while being quantitative and applicable to multiple miRNAs. With its characteristics, DQAMmiR has a potential of becoming the major tool for quantitative analysis of miRNAs *in vitro* for all applications but wide screens.

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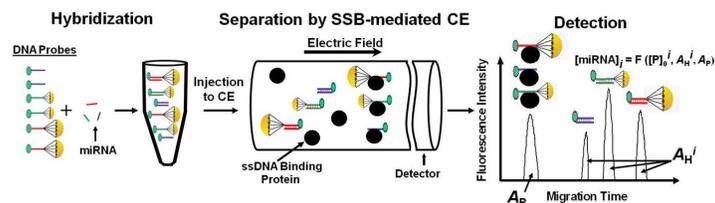
ABSTRACT: Here we report the first direct quantitative analysis of multiple miRNAs (DQAMmiR). DQAMmiR uses miRNAs directly, without chemical or enzymatic modification, and accurately determines concentrations of multiple miRNAs without the need to build calibration curves. In essence, a sample containing multiple miRNAs is mixed with fluorescently-labeled DNA probes complimentary to the miRNAs. After the probes hybridize with miRNAs, the mixture is subjected to gel-free capillary electrophoresis (CE), in which different miRNA-probe hybrids are separated from each other due to different drag tags on the probes, and the separation of the unbound probes from the hybrids is mediated by single strand DNA binding protein that binds the probes but not the hybrids. The concentrations of the miRNAs are calculated using a simple mathematical approach that utilizes the fluorescent signals from the separated hybrids and all unbound probes. In this proof-of-principle work, we developed DQAMmiR for 3 miRNAs (mir21, 125b, 145) known to be deregulated in breast cancer. DQAMmiR opens the opportunity for simple, fast, and quantitative fingerprinting of up to several tens of miRNAs in basic research and clinical applications. The availability of commercial CE instruments suitable for DQAMmiR makes the method practical for a large community of researchers.

SUPPORTING INFORMATION

Direct Quantitative Analysis of Multiple miRNAs (DQAMmiR)

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1. Supporting Material and Methods

1.1. Hybridization Probes. All miRNA for in vitro assays and hybridization probes for mir21, 125b were custom-synthesized by IDT (Coralville, IA). The miRNA had the following sequences: mir21: 5'-UAGCUUAUCAGACUGAUGUUGA-3', mir125b: 5'-UCCCUGAGACCCUAACUUGUGA-3', mir145: 5'-GUCCAGUUUUCCCAGGAAUCCCU-3'. The hybridization probes had the following sequences: mir21 DNA probe: 5'-Alexa488-TCAACATCAGTCTGATAAGCTAGCGCGCTTTGCG CGC-3', mir125b DNA probe : 5'-Alexa 488-TCACAAGTTAGGGTCTCAGGGA-3'. The mir145 DNA probe was synthesized by Sigma-Aldrich (Oakville, ON, Canada): 5'-Alexa 488-AGGGATTC CTGGGAAAACTGGAC-Biotin-3'.

1.2. Hybridization Conditions. Hybridization was carried out in Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Various concentrations of the three miRNA species (mir21, 125b, 145) were incubated with 50 nM of their respective DNA probes along with 1 nM fluorescein (internal standard) in Incubation Buffer (50mM Tris-Ac, 50mM NaCl, 10mM EDTA, pH 7.8). 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 one hour to allow annealing. For the 100 nM miRNA sample the DNA probe concentration was increased to 500 nM and fluoroscein was increased to 100 nM. To minimize miRNA degradation, a nuclease-free environment was used while handling miRNA samples.

1.3. Capillary electrophoresis with fluorescence detection. We used a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA) with laser-induced fluorescence detection. Fluorescence was excited with a 488 nm continuous wave solid-state laser (JDSU, Santa Rosa, CA). 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 9.3, with 50 nM SSB. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, ddH$_2$O and running buffer for one minute each. Samples were injected 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. Electropherograms were analyzed using 32Karat 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, fluorescein. The areas of background impurities (from DNA probes) were subtracted from peak areas of the hybrids. Concentrations of miRNA were determined using equation 1 as explained in the article.

1.4. Quantum yield determination. We took into account the potential change of quantum yield of the DNA probe upon binding to SSB (q_P) and to miRNA (q_H). To determine q_P, 50nM of DNA probe was injected into the capillary (using our hybridization conditions) either with or without a saturating concentration of SSB in the running buffer, 25mM sodium tetraborate, pH 9.3. The quantum yield was determined using the equation S1-1:

$$q_P = \frac{A_P^{[SSB]_{SAT}}}{A_P^{[SSB]=0}}$$

(S1-1)

Where q_P is the relative quantum yield of the respective DNA probe, $A_P^{[SSB]sat}$ is the normalized peak area of the DNA probe in the presence of a saturating concentration of SSB and $A_P^{[SSB]=0}$ is the normalized area of the DNA probe in the absence of SSB. All peak areas were normalized using the internal standard present in sample.

q_H was obtained by injecting two samples of 50nM DNA probe with and without its respective miRNA target, and using equation S1-2:

$$q_H = \frac{A_H}{(A_P^{[miRNA]=0} - A_P^{[miRNA]\neq0})}$$

(S1-2)

Where q_H is the relative quantum yield of the miRNA-DNA probe hybrid, A_H is the normalized area of the hybrid, $A_P^{[miRNA]=0}$ is the normalized area of DNA probe in the absence of target miRNA and $A_P^{[miRNA]\neq0}$ is the normalized area of excess DNA probe in the presence of target miRNA.

1.5. DQAMmiR in cell lysate. An *E.coli* BL21 cell culture was grown to an OD_{600} of 1.6, harvested by centrifugation at 5,000 × g for 10 min at 4 °C and resuspended in sonication buffer: 50 mM Tris-HCl, 2.5 mM $MgCl_2$, 5 mM KCl at pH 8.3. They were lysed by sonication on ice with 5 second ''on''/15 second ''off'' intervals for a total of 10 min. Cell lysates were aliquoted and stored at –80 °C. A 10x dilution of the lysed cells were incubated with 50nM of the three DNA probes, 5nM of each miRNA (mir21, 125b, 145), 20nM fluorescein along with 2.5 µM mask DNA and 1µM mask RNA. Mask DNA was a 20-nucleotide DNA strand, with the sequence of 5'-CAAAAAATGAGTCATCCGGA-3' and the mask RNA was a tRNA library from baker's yeast from Sigma-Aldrich (Oakville, ON, Canada). Hybridization, injection and capillary conditions were performed as previously explained.

MCF-7 cells were purchased from ATCC and grown in incubator at 37°C in the atmosphere of 5% CO_2. Cells were grown in DMEM/F12 media (Invitrogen) with 20 ng/mL hEGF, 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 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 detouch them from bottom of dish and centrifuged at 150×g for 5 min. Pellet was washed twice with PBS. The

cells were counted using haemocytometer and lysed with 1% Triton in 50 mM Tris-Acetate, 50 mM KCl, 10 µM masking RNA, 0.1 mM EDTA, pH 8.16. Cell lysates were aliquoted and stored at –80°C. A 10× dilution of the lysed cells were incubated with 5 nM of the three DNA probes, 0.5 nM of each miRNA (mir21, 125b, 145), 1 nM fluorescein along with 250 nM masking DNA and 100 nM masking RNA. Masking DNA was a 20-nucleotide DNA strand, with the sequence of 5'-CAAAAAATGAGTCATCCGGA-3' and the masking RNA was a tRNA library from baker's yeast from Sigma-Aldrich (Oakville, ON, Canada). Hybridization, injection and capillary conditions were performed as previously explained.

1.6. One-nucleotide differentiation in DQAMmiR. The 1-nucleotide mismatch of mir145 with the sequence 5'-GUCCAGUUUUC**A**CAGGAAUCCCU-3' was custom synthesized by IDT (Coralville, IA). Five nM mir145 or its 1-nucleotide mismatch was incubated with 50 nM mir145 DNA probe and 1 nM fluorescein. Hybridization and injection conditions were performed as previously explained. The capillary temperature was increased to 35°C to allow differentiation.

2. Supporting Results

2.1. Tables of quantum yield values and DQAMmiR-measured miRNA concentrations

Table S1. Quantum yields for 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 the experimental section of the supporting information.

Quantum Yield	Mir145	mir125b	Mir21
q_P	0.29 ± 0.01	0.24 ± 0.02	0.14 ± 0.01
q_H	0.61 ± 0.05	0.63 ± 0.08	0.59 ± 0.06

Table S2. DQAMmiR-determined concentrations of the three miRNA (mir21, 125b, 145) relative to their actual concentration as determined by light absorbance at 260 nm.

Actual miRNA Concentration (nM)	DQAMmiR-Determined miRNA Concentration (nM)		
	mir145	mir125	mir21
0.1	0.091 ± 0.036	0.101 ± 0.016	0.108 ± 0.001
0.5	0.42 ± 0.09	0.49 ± 0.06	0.46 ± 0.04
1	0.84 ± 0.02	1.01 ± 0.03	0.95 ± 0.01
5	4.9 ± 0.1	5.6 ± 0.3	5.3 ± 0.2
10	9.6 ± 0.6	10.3 ± 0.8	10.1 ± 0.4
100	85 ± 5	97 ± 3	92 ± 6

## 2.2. Electropherograms representing dynamic range of DQAMmiR
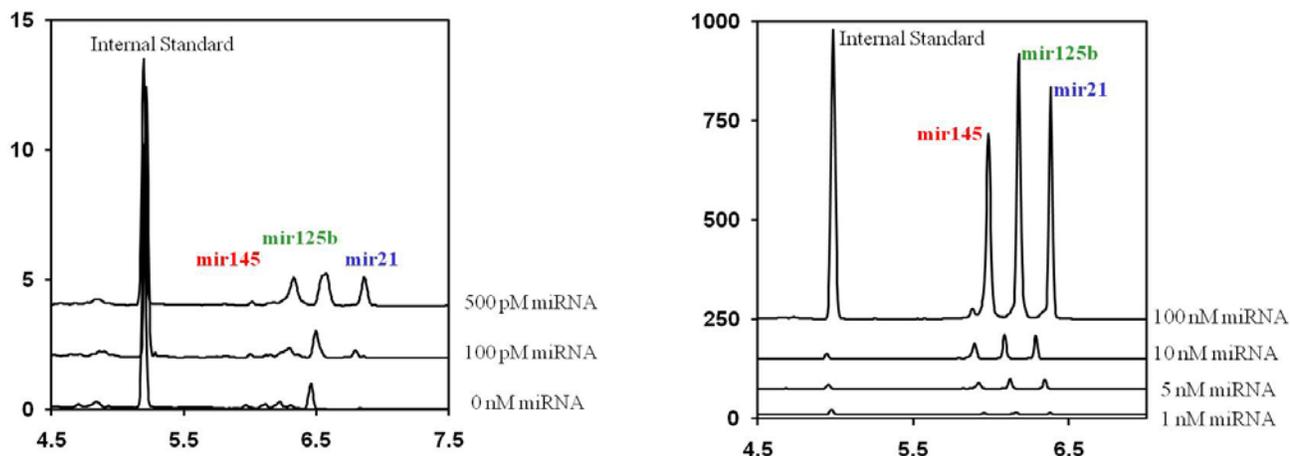
Figure S1. Electropherograms for concentrations of miRNA varying within three orders of magnitude (from 100 pM to 100 nM). All runs (except for 100 nM miRNA) were performed with 50 nM of each of the three DNA probes and 1 nM fluoroscein as the internal standard. The 100 nM miRNA sample contained 500 nM of each of the three DNA probes and 100 nM fluoroscein as the internal standard. The left panel represents 0, 100 and 500 pM miRNA, while the right panel represents 1, 5, 10, and 100 nM miRNA. The areas of the impurity peaks observed in the 0 nM miRNA run, that overlapped with the duplex peaks, were subtracted from the normalized miRNA peak areas.

2.3. Table and figure for results on DQAMmiR-measured miRNA added to *E. coli* lysate

Table S3. A comparison of miRNA concentrations determined by DQAMmiR with and without BL21 cell lysate. Five nM of each miRNA (145, 125b, 21) were incubated with: a) 50 nM DNA probes (145, 125b, and 21), 20 nM fluorescein or b) 50 nM DNA probes (145, 125b, 21), 20 nM fluorescein, 10 times diluted BL21 *E. coli* lysate, 2.5 µM masking DNA, and 1 µM masking RNA. Each miRNA had comparable results with and without the cell lysate and masking DNA/RNA.

Sample	DQAMmiR-determined miRNA concentration (nM)		
	mir145	mir125	mir21
5 nM each miRNA	5.15 ± 0.24	4.52 ± 0.22	5.33 ± 0.25
5 nM each miRNA + Masking DNA/RNA + *E. coli* cell lysate	5.15 ± 0.20	4.52 ± 0.18	5.08 ± 0.16
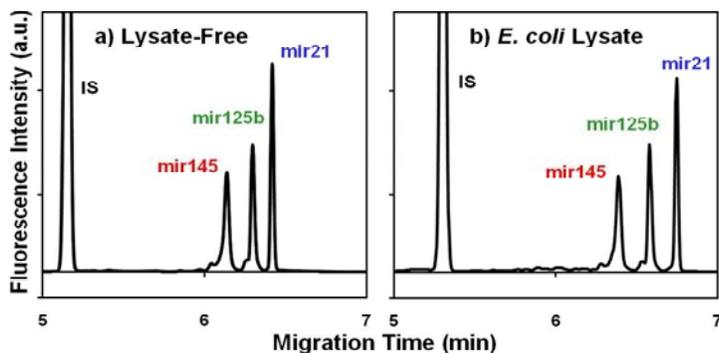
Figure S2. The influence of complex biological matrix on miRNA analysis by DQAMmiR. **a)** DQAMmiR of 3 miRNAs at 5 nM each in *E. coli* lysate and masking DNA/RNA in incubation buffer. **b)** DQAMmiR of 3 miRNAs at 5nM each in pure incubation buffer. IS labels the peak of the internal standard (fluorescein).

2.4. One-nucleotide differentiation
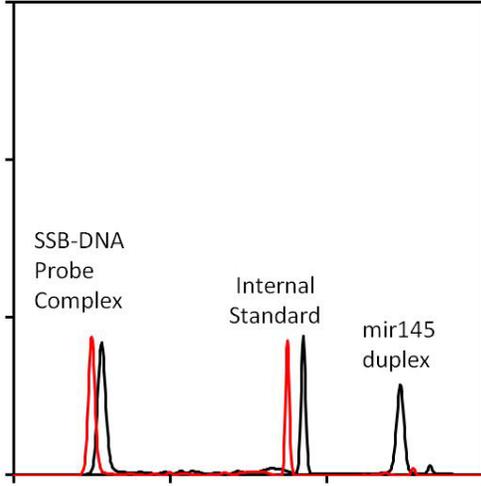
Figure S3. Electropherogram representing 1-nucleotide differentiation of the mir145 DNA probe using DQAMmiR. Five nM mir145 (black) or 5 nM 1-nucleotide mismatch mir145 (red) were incubated with 50 nM mir145 DNA probe and 1 nM fluorescein. For each run the capillary temperature was increased to 35°C. At this temperature there was no 1-nucleotide mismatch duplex peak while the mir145 duplex peak remained intact.

3. Supporting Mathematics (derivation of equation for the determination of concentrations of multiple miRNAs in DQAMmiR)

The unknown concentration of the i-th miRNA, $[\text{miRNA}]^i$, can be expressed through the area of its respective hybrid peak (A_H^i), using the unknown coefficient a and known quantum yield q_H^i:

$$[\text{miRNA}]^i = a\left(A_\text{H}^i / q_\text{H}^i\right) \tag{S3-1}$$

The known concentration of the i-th probe, $[\text{P}]_0^i$, can be expressed through the areas of two peaks, the one of SSB-bound excess probe, A_P^i, and the one of the miRNA-bound probe, A_H^i, with the same coefficient a and known quantum yields q_H^i and q_P^i:

$$[\text{P}]_0^i = a\left(A_\text{H}^i / q_\text{H}^i + A_\text{P}^i / q_\text{P}^i\right) \tag{S3-2}$$

Accordingly, the known total concentration of N DNA probes can be expressed using the following equation:

$$\sum_i^N [\text{P}]_0^i = a\left(\sum_i^N A_\text{H}^i / q_\text{H}^i\right) + a\left(\sum_i^N A_\text{P}^i / q_\text{P}^i\right) \tag{S3-3}$$

Since the peaks of the hybrids are resolved, their corresponding areas A_H^i can be experimentally determined; accordingly we treat them as known parameters. The peaks corresponding to the SSB-bound excess probes can, however, overlap. Therefore, we treat the areas corresponding to them, A_P^i, as unknowns along with the coefficient a. While the individual A_P^i are unknown, their sum, A_P, can be experimentally measured and can thus be treated as a known parameter. To incorporate A_P in the equation, we need to isolate A_P^i from q_P^i by multiplying Equation S3-3 by q_P^i:

$$\sum_{i}^{N} [P]_0{}^i q_P{}^i = a\left(\sum_{i}^{N} A_H{}^i q_P{}^i / q_H{}^i\right) + a\left(\sum_{i}^{N} A_P{}^i\right) \tag{S3-4}$$

Equation S3-4 can be otherwise represented as:

$$\sum_{i}^{N} [P]_0{}^i q_P{}^i = a\left(\sum_{i}^{N} A_H{}^i q_P{}^i / q_H{}^i\right) + a A_P \tag{S3-5}$$

Now we can solve Equation S3-5 for a:

$$a = \frac{\displaystyle\sum_{i}^{N} [P]_0{}^i q_P{}^i}{\left(\displaystyle\sum_{i}^{N} A_H{}^i q_P{}^i / q_H{}^i\right) + A_P} \tag{S3-6}$$

By expressing a from Equation S3-1 and incorporating it into Equation S3-6 we get:

$$\frac{[\mathrm{miRNA}]^i q_H{}^i}{A_H{}^i} = \frac{\displaystyle\sum_{i}^{N} [P]_0{}^i q_P{}^i}{\left(\displaystyle\sum_{i}^{N} A_H{}^i q_P{}^i / q_H{}^i\right) + A_P} \tag{S3-7}$$

We can finally express the unknown concentration of the i-the miRNA in the following way:

$$[\mathrm{miRNA}]^i = \frac{A_H{}^i \displaystyle\sum_{i}^{N} [P]_0{}^i q_P{}^i}{q_H{}^i \left[\left(\displaystyle\sum_{i}^{N} A_H{}^i q_P{}^i / q_H{}^i\right) + A_P\right]} \tag{S3-8}$$