analytical chemistry

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Achieving Single-Nucleotide Specificity in Direct Quantitative Analysis of Multiple MicroRNAs (DQAMmiR)

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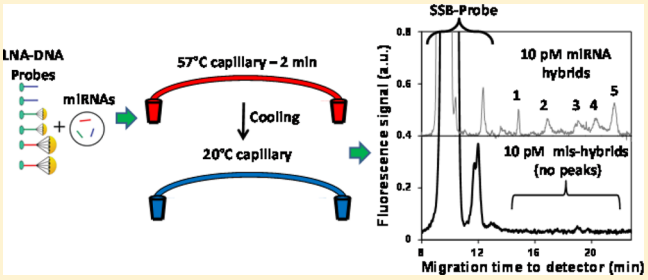
Supporting Information

ABSTRACT: Direct quantitative analysis of multiple miRNAs (DQAMmiR) utilizes CE with fluorescence detection for fast, accurate, and sensitive quantitation of multiple miRNAs. Here we report on achieving single-nucleotide specificity and, thus, overcoming a principle obstacle on the way of DQAMmiR becoming a practical miRNA analysis tool. In general, sequence specificity is reached by raising the temperature to the level at which the probe-miRNA hybrids with mismatches melt while the matches remain intact. This elevated temperature is used as the hybridization temperature. Practical implementation of this apparently trivial approach in DQAMmiR has two major challenges. First, melting temperatures of all mismatched hybrids should be similar to each other and should not reach the melting temperature of any of the matched hybrids. Second, the elevated hybridization temperature should not deteriorate CE separation of the hybrids from the excess probes and the hybrids from each other. The second problem is further complicated by the reliance of separation in DQAMmiR on single-strand DNA binding protein (SSB) whose native structure and binding properties may be drastically affected by the elevated temperature. These problems were solved by two approaches. First, locked nucleic acid (LNA) bases were incorporated into the probes to normalize the melting temperatures of all target miRNA hybrids allowing for a single hybridization temperature; binding of SSB was not affected by LNA bases. Second, a dual-temperature CE was developed in which separation started with a high capillary temperature required for proper hybridization and continued at a low capillary temperature required for quality electrophoretic separation of the hybrids from excess probes and the hybrids from each other. The developed approach was sufficiently robust to allow its integration with sample preconcentration by isotachophoresis to achieve a limit of detection below 10 pM.



MiRNAs are 18−25 nucleotide, noncoding, RNA sequences that play a significant role in gene regulation. They are involved in major cellular processes including differentiation, apoptosis, and proliferation.[1−5] The deregulation of specific miRNA species has been found to correlate with significant human diseases, such as heart disease,[6] Alzheimers disease,[7] and cancer.[8,9] These subsets, termed miRNA "fingerprints", have great potential as diagnostic biomarkers. Importantly, the discovery of cancer-specific fingerprints in biological fluids (blood, urine) has shown that the detection of deregulated miRNA can potentially be used to diagnose early stage cancers by noninvasive methods[10,11] and may help to guide subsequent patient management.[12] Quantitative miRNA fingerprints can be potentially used for cancer subtyping.[8,13] The quantitative analysis of miRNA fingerprints requires a method capable of accurate quantitation of multiple miRNAs. Methods for miRNA analysis have been recently reviewed and classified.[14] Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)[15,16] and next-generation sequencing[17] are two of the most common miRNA detection techniques; however, they are indirect, meaning that they require modification or amplification of the target miRNA, which are prone to sequence-related biases. Recently, miRNA detection with the use of mass-spectrometry was demonstrated; the method is indirect as it requires biotinylation of the target miRNA.[18] Nanostring technology,[19] although direct, currently is not sensitive enough to detect low abundance miRNA and is time-consuming and cost-prohibitive in routine analyses. There

Received: December 10, 2015
Accepted: January 12, 2016
Published: January 12, 2016

DOI: 10.1021/acs.analchem.5b04682
Anal. Chem. 2016, 88, 2472−2477

have been several recently introduced direct miRNA detection methods;[14,20−28] however, they are still in their early stages of development and are not currently applicable to a clinical setting.

We recently introduced a CE-based approach for direct, quantitative analysis of multiple miRNAs (Figure 1), termed

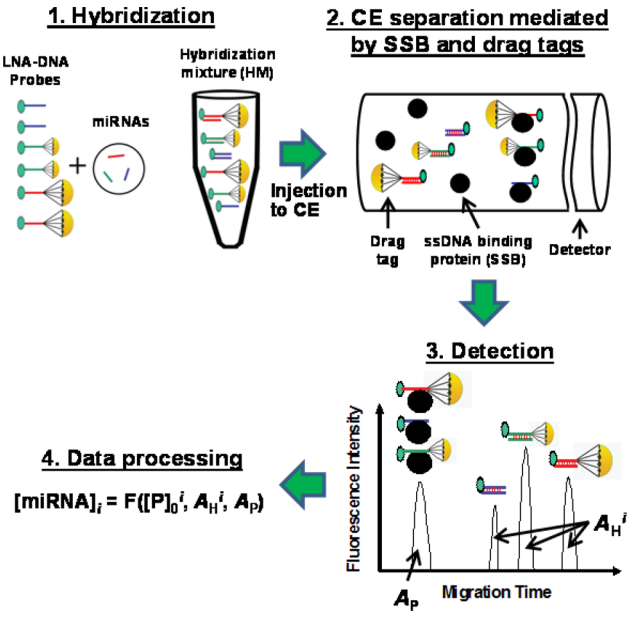


Figure 1. Schematic representation of the four major steps in DQAMmiR.

DQAMmiR.[29] DQAMmiR is a hybridization assay that uses miRNA-specific DNA probes, labeled with a fluorophore for laser-induced fluorescence (LIF) detection. Straightforward CE cannot separate the miRNA-DNA hybrids from each other and from the excess probes as all of them have similar charge to size ratios. We resolved this problem by adding single-strand DNA binding protein (SSB) to the run buffer and by modifying probes with drag tags. SSB binds the unbound DNA probes and separates them from the hybrids. The different drag tags change the size to charge ratios of the hybrids and allow their separation from each other (Figure 1).[26]

In our proof of principle work, we quantified 3 miRNAs simultaneously from a biological sample with a detection limit of 100 pM.[29] In further experiments, we were able to (i) lower assay time down to 20 min by optimizing the DNA probe concentration and purity,[30] (ii) simultaneously detect 5 target miRNA species by conjugating peptide drag tags to the DNA probes allowing for hybrid peak separation,[31] and (iii) significantly improve the limit of detection (LOD) by incorporating isotachophoresis (ITP) into our runs.[32] ITP is an in-capillary preconcentration technique that, when combined with DQAMmiR, allows for the detection of multiple miRNAs down to a concentration of 1 pM. With these improvements we have made DQAMmiR into a fast, sensitive, quantitative miRNA detection method capable of detecting multiple miRNAs from biological samples in a robust, cost-effective fashion.

The remaining limitation of DQAMmiR, however, was the inability to achieve single nucleotide specificity for multiple miRNAs. Achieving such specificity with DQAMmiR is not a trivial matter, requiring both the ability to dissociate all respective mismatches from their respective probes at a single temperature and the maintenance of this temperature at the locations of the capillary where mismatches and DNA probes are both present and able to interact.

We resolved these issues by (i) introducing locked nucleic acid (LNA) bases to the DNA probes, designed to equalize the melting temperatures of all hybrids and (ii) a dual-temperature technique designed to support proper hybridization at the injection end of the capillary while using a lower temperature during the main part of CE separation. The introduction of these allowed us to achieve single-nucleotide (miRNA analogs differing by 1-nt) specificity while maintaining the ability to separate and accurately quantitate multiple miRNAs. We also were able to combine this approach with sample preconcentration by ITP.

EXPERIMENTAL SECTION

Hybridization Probes and miRNAs. All miRNAs and mismatch RNAs were custom-synthesized by IDT (Coralville, IA, USA). All LNA probes were custom-synthesized by Exiqon (Woburn, MA, USA), with a 3′ FAM (6-fluorescein amidite) for detection and a 5′ thiol group for conjugation with peptide drag tags. To allow separation of the 5 hybrids, 4 peptide drag tags of varying lengths were conjugated to the LNA-DNA probes via a thioether bond. The conjugation reaction, which is described in our previous work,[31] occurred between the thiol group on the 5′ end of the LNA-DNA probes and a maleimide group on the N-terminus of the peptide drag tags. All maleimide modified peptides were synthesized by Canpeptide (Pointe-Clare, QC, Canada). The sequences of all miRNAs, mismatch RNAs, LNA-DNA probes, and peptides can be found in Supporting Table S1.

Hybridization Conditions. Hybridization was carried out in a Mastercycler 5332 thermocycler (Eppendorf, Hamburg, Germany). Various concentrations of the five miRNA species (miR10b, miR21, miR125b, miR145, and miR155) were incubated with 500 nM of their respective DNA or LNA-DNA probes along with 10 nM fluorescein (internal standard) in incubation buffer (50 mM Tris-acetate, 50 mM NaCl, 10 mM EDTA, pH 8.2). Temperature was increased to a denaturing 80 °C and then lowered to 57 °C at a rate of 20 °C/min and was held at 57 °C for 10 min to allow hybridization. To minimize miRNA degradation, a nuclease-free environment was used while handling miRNA samples.

DQAMmiR. We used a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter, Fullerton, CA) with laser-induced fluorescence detection. We used bare fused-silica capillaries with an outer diameter of 365 μm, an inner diameter of 75 μm, and a total length of 79.4 cm. The distance from the injection end of the capillary to the fluorescence detector was 69.0 cm. The temperature was set at 37 °C when using DNA probes and 57 °C when using LNA-DNA probes. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, deionized H_2O and run buffer (25 mM Borax, pH 9.2 containing 100 nM SSB) for 1 min each. Samples were injected by a pressure pulse of 0.5 psi (3.45 kPa) for 5 s. The volume of the injected sample was 14 nL. Electrophoresis was driven by an electric field of 378 V/cm (positive electrode at the injection end). Laser-induced fluorescence of the FAM label was used for detection; a continuous wave solid-state laser emitting at 488 nm (JDSU, Santa Rosa, CA, USA) was used as an excitation source. Electropherograms were analyzed using 32 Karat Software. For dual-temperature DQAMmiR the capillary was

DOI: 10.1021/acs.analchem.5b04682
Anal. Chem. 2016, 88, 2472−2477

set at 57 °C for the first 2 min after injection of the sample. The voltage was then shut off, and the capillary was allowed to cool down to 20 °C (which took 7 min). Once the capillary temperature reached 20 °C the voltage was reapplied and the run continued until the samples passed the detector.

ITP-DQAMmiR. The ITP-DQAMmiR experiments were performed using the same length capillary as previously mentioned with an initial temperature of 20 °C. The capillary was flushed prior to every CE run with 0.1 M HCl, 0.1 M NaOH, deionized H_2O, and TE buffer (20 mM Tris, 10 mM HEPES, pH 8.3) for 1 min each. Varying concentrations of target miRNAs or the 1-nt mismatches were incubated with 10 nM of their respective LNA-DNA probes and were injected from the outlet end by a pressure pulse of 3.0 psi (20.7 kPa) for 99 s. The volume of the injected sample was 1.9 μL. The buffer in the outlet end was switched to LE buffer (50 mM Tris-Cl, 10 mM NaCl, pH 8.0), and an electric field was applied in the reverse direction (negative electrode at the injection end). Electrophoresis was driven by an electric field of 378 V/cm. The voltage was turned off at $t_{cr} - 10$ s, where t_{cr} is the predetermined "critical time-point" explained in our previous work.[31] The capillary was allowed to heat up to 57 °C (which took 8 min) and the buffer in the inlet end was switched to LE supplemented with 100 nM SSB. An electric field of 378 V/cm was applied in the forward direction (positive electrode at the injection end) for 2 min at 57 °C. The voltage was then shut off, and the capillary was allowed to cool to 20 °C within 7 min. Once the capillary temperature reached 20 °C the voltage was reapplied and the run continued until the electrophoretic zones of all miRNA-probe hybrids passed the detector.

Quantitation of miRNA. 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. Concentrations of miRNA were determined using the following equation:

$$[\text{miRNA}]_i = \frac{A_{H,i}}{A_P + \sum_{j=1}^{N} (q_{P,j}/q_{H,j}) A_{H,j}} \left(\sum_{j=1}^{N} (q_{P,j}/q_{H,i}) [P]_{0,j} \right)$$

where $[P]_{0,j}$ is the total concentration of the jth probe (composed of the hybrid and the miRNA-unbound probe), A_H is the area corresponding to the ith or jth hybrid, A_P is the cumulative area of the excess probe, q_H is the relative quantum yield of the ith or jth hybrid with respect to that of the free probe, $q_{P,j}$ is the relative quantum yield of the jth probe in the presence of SSB with respect to that in the absence of SSB, and N is the total number of probes. In this equation, we assume that all target miRNAs are fully hybridized due to the excess of the probes. Quantum yields can be found in Tables S2–S4. See the Supporting Information for the derivation of the equation.

Determination of miRNA Concentration using UV Absorbance. For Figure 4, the "actual concentration" of the target miRNA was determined using UV absorbance. The light absorbance for each miRNA stock solution was measured at 260 nm using the Nano-Drop ND-1000 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). The concentration was calculated using the Beer's law and the miRNA's molar extinction coefficient provided by IDT.

RESULTS AND DISCUSSION

An inherent problem with any miRNA hybridization assay is that there is a wide variance in melting temperatures for the different miRNA-DNA hybrids. Each DNA probe has a specific temperature at which target miRNA binding occurs while nonspecific binding does not. Thus, for the detection of all miRNA with 1-nt specificity a single hybridization temperature should be used. To solve this problem, we incorporated LNA bases into our DNA probes. LNA bases are modified RNA nucleotides, that are "locked" into a conformation that enhances base stacking, thus improving the strength of hybridization. The addition of a single LNA base can increase the melting temperature of a hybrid by 2–4 °C.[33] By varying the number of LNA bases in each of the DNA probes we could equalize the melting temperatures of all the respective hybrids, allowing for 1-nt sensitivity of multiple miRNAs.[33] LNA bases were previously used to achieve 1-nt sensitivity in analyses of multiple miRNA.[34,35] We obtained LNA-DNA probes from Exiqon, with each probe having an RNA-specific melting temperature of 83 °C. It is known that SSB cannot bind to LNA oligonucleotides,[36] however, the LNA bases were shown to have no detrimental effects on the LNA-DNA probe's ability to bind to SSB as there were a sufficient number of DNA bases in each probe to allow SSB binding (data not shown).

We first had to find the hybridization temperature that allowed for differentiation between the miRNA and a 1-nt mismatch for each LNA-DNA probe. Commonly this is estimated to be 30 °C below the melting temperature.[37] When determining the optimum temperature we also had to consider the loss of peak resolution in CE due to peak broadening, caused by increasing the capillary temperature.[38] With this taken into account the optimum temperature was defined as the lowest temperature at which differentiation between the miRNA and a 1-nt mismatch occurred. To determine the optimum temperature we gradually increased the capillary temperature until we reached the point (which we found to be 57 °C) at which the 1-nt mismatch could no longer bind to the LNA probe causing an absence of the respective "mis-hybrid" peak. We then confirmed that the miRNA could still fully bind to the LNA probe at 57 °C by comparing peak areas with the miRNA-probe hybrid at our typical ambient temperature of 20 °C (Figure S1). It should be noted that we could not just incubate the sample at 57 °C prior to its injection into a capillary at 20 °C. We found that the hybridization of LNA-DNA probes to miRNA was so fast that the probes began binding to the mismatches inside the capillary even when a voltage was applied immediately after sample injection (with a 15-s instrument-induced delay). Thus, we required the capillary to be set at 57 °C, which would maintain the dissociation between the probes and their respective 1-nt mismatches. With the optimum hybridization temperature determined we compared the specificity of multiple miRNAs both with LNA-DNA probes and with DNA probes. When using DNA probes (and a predetermined optimum temperature for one of the miRNA hybrids) the differentiation of even as few as two miRNAs vs their respective 1-nt mismatches could not be achieved (Figure 2A). With the LNA-DNA probes, when the capillary was set at 57 °C, we were able to differentiate both miRNAs from their respective mismatches, even with the mismatches at a concentration 10 times higher than that of the miRNAs (Figure 2B). This confirmed that equalizing the melting temperature is vital for specificity of analysis of multiple miRNAs.

Though the use of a melting temperature of 83 °C and a hybridization temperature of 57 °C gave us successful results, we do not imply that these specific temperatures are universal or optimal. Lowering the melting temperature (by incorporat-

DOI: 10.1021/acs.analchem.5b04682
Anal. Chem. 2016, 88, 2472–2477

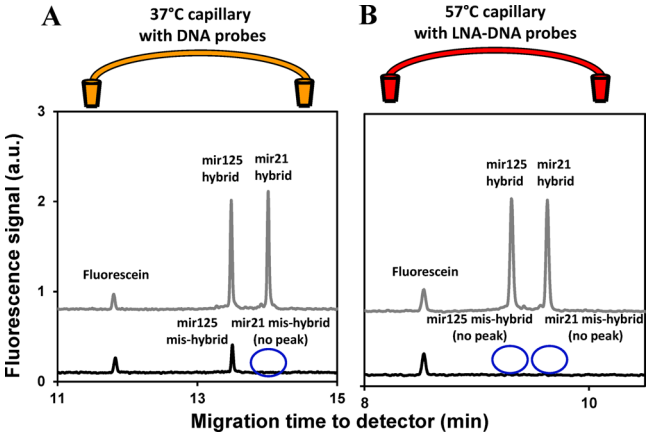


Figure 2. Single-nucleotide specificity of two miRNA-specific probes consisting of DNA (A) or DNA-LNA (B). Panel A: Two DNA-only probes were incubated with 5 nM of the two respective miRNAs, mir21 and mir125b (gray trace) or 50 nM of the 1-nt mismatches (1-nt) of the two miRNAs (black trace) at 37 °C. Single-nucleotide specificity of multiple miRNA was not achieved as seen by presence of the mir125b mis-hybrid peak. 37 °C was the optimum temperature for mir21 specificity. Panel B: Two DNA-LNA probes were incubated with 5 nM of the two respective miRNAs, mir21 and mir125b (gray trace) or 50 nM of the 1-nt mismatches of the two miRNAs (black trace) at 57 °C. Inclusion of LNA bases allowed for 1-nt specificity as seen by the absence of the mis-hybrid for both mir125 and mir21 probes. 57 °C was the optimum temperature for both mir21 and mir125b with their respective LNA-DNA probes. Blue circles indicate where peaks would appear if present.

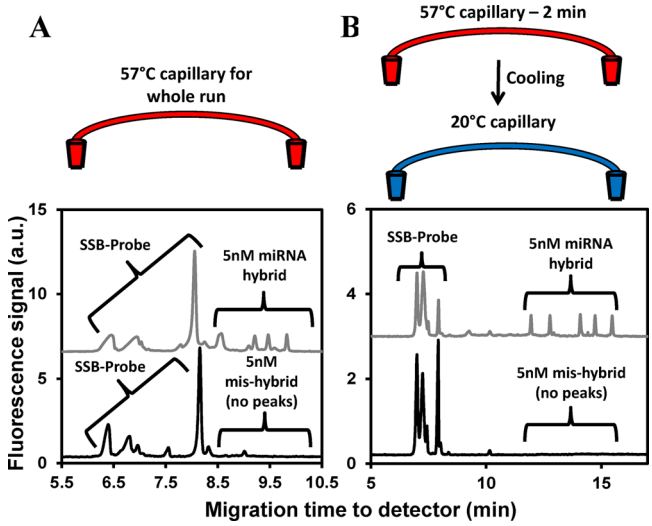


Figure 3. Separation and detection of 5 miRNA-probe hybrid peaks with a 57 °C capillary (A) and by using the dual temperature technique (B). Panel A: With a capillary at 57 °C, 5 LNA-DNA probes were incubated with their respective miRNAs (gray trace) or with 1-nt mismatches of the 5 miRNAs (black trace). Single-nucleotide specificity was achieved for all 5 miRNAs as observed by the absence of 1-nt mis-hybrid peaks. Though specificity was achieved, 57 °C caused a loss of resolution and a lack of SSB-probe binding, preventing the separation and detection of all 5 miRNA hybrids. Panel B: Using a dual temperature technique, 5 LNA-DNA probes were incubated with their respective miRNAs (gray trace) or with 1-nt mismatches of the 5 miRNAs (black trace). The capillary was set at 57 °C for the first 2 min of the run to allow 1-nt specificity. The capillary is then cooled down to 20 °C to allow proper SSB-binding and separation of the 5 hybrid peaks. Using the dual-temperature technique the separation of all 5 hybrid peaks is observed while 1-nt specificity is maintained.

ing fewer LNA bases in the DNA probe) may potentially be beneficial as this can improve SSB binding and reduce peak broadening. On the other hand, lowering the melting temperature should not raise any major issues, as all mismatch hybrids should theoretically have lower melting temperatures than the target miRNA hybrids. The one caveat (regardless of the set melting temperature) is that 1-nt mismatch binding can vary, depending on its sequence and location of the mismatch, making it difficult to determine accurately how close the melting temperature of its hybrid is to that of the target miRNA hybrid. As such, 1-nt specificity must be tested for each new set of target miRNAs.

Placing LNA bases at the location of known mismatches (such as close family members) can help increase the difference in melting temperature between the target miRNA hybrid and its respective mismatch hybrid.[38] This makes it possible to achieve 1-nt specificity regardless of mismatch location.[34,39] Thus, locating LNA bases in immediate proximity of known 1-nt mismatches seems to be a promising strategy for future work.

The next step was to determine whether we could differentiate 5 different miRNA hybrids from their respective 1-nt mis-hybrids, using the LNA-DNA probes and the capillary temperature of 57 °C. To allow sufficient separation of the 5 hybrid peaks we required the use of peptide drag tags with lengths of 0, 5, 10, 15, and 20 amino acids, as in our previous work.[31] Similar to our experiments with 2 miRNAs (Figure 2B), we successfully differentiated all 5 miRNAs from their mismatches. All 5 miRNA peaks were present, while no mismatch peaks were observed (Figure 3A). This showed that the 57 °C hybridization temperature was optimal for all 5 probe-miRNA hybrids. This achievement was accompanied, however, by a significant loss in resolution, with very little separation between the hybrid peaks and the excess LNA-DNA probes. The loss of resolution was expected as increased capillary temperatures caused peak broadening.[40] Also, a worsening of SSB binding was apparent, with the shift of the LNA-DNA probes back toward the hybrid peaks. We needed to resolve this issue as the lack of separation limited the number of detectable miRNAs and made it difficult to accurately quantitate the peak areas of each of the hybrids. We hypothesized that the 57 °C dissociation temperature was required only in the beginning of separation to minimally separate the hybrids from the excess probes while preventing reassociation of the probes with mismatches. This initial separation was possible due to residual binding of SSB to the probes even at this high temperature. Based on the velocities of the hybrids and the SSB-bound probes, we calculated that there was sufficient separation between the RNA and SSB-bound LNA-DNA probes to prevent reassociation after 2 min under our separation conditions. Thus, if we maintained the capillary temperature at 57 °C for the first 2 min, the capillary temperature could be reduced to 20 °C for the rest of separation to facilitate full-strength SSB binding and hybrid separation. With this dual-temperature technique we were able to achieve a resolution sufficient for the detection of 5 miRNA hybrids, while still maintaining 1-nt specificity (Figure 3B).

Our final step was to ensure that we could still achieve 1-nt specificity and sufficient hybrid separation with in-capillary preconcentration of the sample with ITP. Briefly, a very long plug of the hybridization mixture was injected into the capillary from its outlet end of the capillary. The buffers with different

DOI: 10.1021/acs.analchem.5b04682
Anal. Chem. 2016, 88, 2472−2477

conductivities were used in the inlet and outlet reservoirs. The components of the hybridization mixture were focused at the interface between a high conductivity leading electrolyte (LE) and a low conductivity trailing electrolyte (TE), increasing the local sample concentration by 2 orders of magnitude.[32] Once the sample reached the inlet end of the capillary, the inlet and outlet ends of the capillary were placed in vials with LE supplemented with 100 nM SSB; the electric field was reversed, and DQAMmiR was run toward the detector. The introduction of the dual-temperature technique did not interfere with ITP's ability to preconcentrate our samples. Furthermore, having the capillary temperature at the required hybridization temperature allowed us to incubate the sample inside the capillary after ITP. This reduced the overall assay time as the increase in concentration of the sample significantly reduced the required incubation time.[30] We performed ITP according to our previous protocol,[32] and once the sample reached the end of the capillary we increased the capillary temperature to 57 °C. A separation voltage was applied and DQAMmiR was performed using the dual-temperature technique allowing for separation and detection of the 5 hybrids at the low pM concentrations (Figure 4A). Thus, we achieved 1-nt specificity of multiple

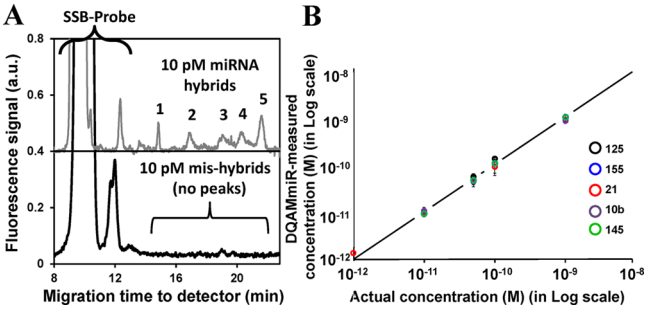


Figure 4. Applying dual-temperature technique to DQAMmiR with ITP preconcentration. Panel A: Detection of either 10 pM of 5 miRNAs (gray trace) or 10 pM of their respective 1-nt mismatches using the ITP-DQAMmiR tandem. Labels 1−5 indicate the 5 hybrid peaks from left to right corresponding to mir10b, mir155, mir145, mir125b, and mir21. Panel B: Quantitation of the 5 respective miRNAs over a range of concentrations from 1 pM to 1 nM.

miRNA while maintaining high quality separation and low LOD. It should be noted that ITP in its nature is very sensitive to its buffer composition. Even slight changes in buffer concentration, pH or temperature can significantly affect the result. Optimization of all buffers is required with any parameter changes including temperature, voltage, and sample concentration. A detailed explanation of ITP buffer optimization can be found in our previous work.[32] With this in mind, we were still able to use this technique over multiple days with multiple users and achieved reproducible results over a range of concentrations (Figure 4B).

CONCLUSIONS

In conclusion, we successfully achieved 1-nt specificity while detecting multiple miRNAs simultaneously by the DQAMmiR method. LNA bases were introduced into the DNA probes to equalize the respective hybrid's melting temperatures. At a temperature of 57 °C all 5 miRNA were able to bind to their respective probes while the 1-nt mismatches could not. The use of the dual-temperature technique allowed us to achieve proper resolution of hybrid peaks while maintaining 1-nt specificity. This technique works with our recently introduced ITP-DQAMmiR combination, allowing us to have great specificity and sensitivity in a single run. The use of LNA bases and dual-temperature CE allowed us to achieve 1-nt specificity with DQAMmiR using an automated, commercially available instrument, which makes this approach suitable for use in a clinical setting.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04682.

LNA-DNA, miRNA, and peptide sequences, supporting tables and electropherograms for optimization of capillary temperatures (PDF)

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Author Contributions

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

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Canadian Institute of Health Research and Natural Sciences and Engineering Research Council of Canada.

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DOI: 10.1021/acs.analchem.5b04682
Anal. Chem. 2016, 88, 2472−2477

SUPPORTING INFORMATION

Achieving Single-Nucleotide Specificity in Direct Quantitative Analysis of Multiple MicroRNAs (DQAMmiR)

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Table of Contents

1. SUPPORTING MATERIALS AND METHODS

Table S1. List of target miRNAs, their nucleotide sequences, their respective single nucleotide mismatch sequence with mismatch highlighted in red, sequences of corresponding LNA-DNA hybridization probes and their respective peptide drag tags. As it is proprietary information to Exiqon, we do not know the locations or the number of LNA bases in each probe. We did ensure that there were multiple LNA-free stretches of at least 3 DNA bases in each probe to allow proper SSB binding.

Name of sequence	**miRNA Nucleotide sequence**	**Single nucleotide mismatch sequence**	**Hybridization probe sequence with modifications**	**Peptide drag tag sequence**
mir-21	5'-UAG CUU AUC AGA CUG AUG UUG A-3'	5'-UAG CUU AUC A**U**A CUG AUG UUG A-3'	5'-ThiolC6S-S-TCA ACA TCA GTC TGA TAA GCT A-FAM-3'	none
mir-125b	5'-UCC CUG AGA CCC UAA CUU GUG A-3'	5'-UCC CUG AGA **A**CC UAA CUU GUG A-3'	5'-ThiolC6S-S-TCA CAA GTT AGG GTC TCA GGG A-FAM-3'	C-term-Gly-Ala-Gly-Thr-Gly-N term
mir-145	5'-GUC CAG UUU UCC CAG GAA UCC CU-3'	5'-GUC CAG UUU UC**A** CAG GAA UCC CU-3'	5'-Thiol C6S-S-AGG GAT TCC TGG GAA AAC TGG AC-FAM-3'	C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term
mir-155	5'-UUA AUG CUA AUC GUG AUA GGG GU-3'	5'-UUA AUG CUA AUC **C**UG AUA GGG GU-3'	5'-ThiolC6S-S-ACC CCT ATC ACG ATT AGC ATT AA-FAM-3'	C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term
mir-10b	5'-UAC CCU GUA GAA CCG AAU UUG UG-3'	5'-UAC CCU GUA GA**A** CCG AAU UUG UG-3'	5'-ThiolC6S-S CAC AAA TTC GGT TCT ACA GGG TA-FAM-3'	C-term-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-Gly-Ala-Gly-Thr-Gly-N term

2. SUPPORTING RESULTS

To accurately quantitate our target miRNA, we have to take into consideration i) the potential influence SSB-binding has on the quantum yield of our LNA-DNA probes (q_P) and ii) the potential influence miRNA-binding has on the quantum yield of the LNA-DNA probe-miRNA hybrids (q_H). In our previous work [29] we found that both SSB-binding and miRNA binding both influence the quantum yields of the probes. Furthermore, we also had to take into account the potential influence the conjugation of peptides would have on our LNA-DNA probe quantum yields (q_D) which we had previously shown. [31] Finally, we had to multiply both q_P and q_H by q_D to take into account the loss of signal from both SSB/miRNA binding and from the conjugation to the peptides respectively. Such quantum yields are shown in the tables below and in the Supporting Mathematics.

2.1. Tables of quantum yields and DQAMmiR-measured miRNA concentrations

Table S2. 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 of the DNA probe-miRNA hybrid. These values were determined as explained in our previous work.[29]

DNA Probe Type	MiR10b DNA probe	MiR155 DNA probe	MiR145DNA probe	MiR125b DNA probe	MiR21 DNA probe
q_P	0.54 ± 0.05	0.84 ± 0.06	0.3 ± 0.03	0.7 ± 0.04	0.26 ± 0.02
q_H	0.37 ± 0.03	0.33 ± 0.03	0.23 ± 0.01	0.51 ± 0.03	0.66 ± 0.04

Table S3. After the conjugation of peptides to the miRNA-specific DNA probes (peptide lengths of 5, 10, 15, and 20 amino acids were conjugated to the DNA probes for miR125b, miR145, miR155, and miR10b, respectively), the variation of fluorescence intensity was taken into account. The fluorescence intensity of all LNA-DNA probes was normalized by determining their quantum yields (q_D) with respect to an untagged LNA-DNA probe (the untagged probe for miR21 was used as a reference).

DNA Probe Type	MiR10b-20aa DNA probe	MiR155-15aa DNA probe	MiR145-10aa DNA probe	MiR125b-5aa DNA probe	MiR21 DNA probe
q_D	0.54 ± 0.02	0.59 ± 0.04	0.40 ± 0.02	0.91 ± 0.06	1

Table S4. Quantum yields of DNA probes conjugated to peptides upon binding to SSB (q_P') and upon hybridization with miRNA (q_H'). They were obtained by multiplying q_P and q_H by q_D

DNA Probe Type	MiR10b-20aa DNA probe	MiR155-15aa DNA probe	MiR145-10aa DNA probe	MiR125b-5aa DNA probe	MiR21 DNA probe
q_P'	0.29 ± 0.02	0.49 ± 0.02	0.12 ± 0.02	0.64 ± 0.02	0.26 ± 0.02
q_H'	0.20 ± 0.02	0.19 ± 0.02	0.09 ± 0.01	0.46 ± 0.02	0.66 ± 0.01

Table S5. DQAMmiR-determined concentrations of the five miRNA (mir125b, mir155, mir21, mir10b, mir145) relative to their actual concentration as determined by light absorbance at 260 nm.

Actual miRNA Concentration (pM)	DQAMmiR-Determined miRNA Concentration (pM)				
	Mir10b	mir155	Mir145	mir125b	Mir21
1	-	-	-	-	1.23 ± 0.39
10	10.1 ± 0.46	9.78 ± 2.3	8.8 ± 0.29	8.90 ± 1.1	9.15 ± 0.81
50	43.6 ± 6.7	45.3 ± 12	46.4 ± 4.8	55.5 ± 9.0	47.1 ± 9.3
100	106 ± 22	91.5 ± 33	108 ± 42	136 ± 4.2	90.9 ± 25
1000	901 ± 115	1094 ± 129	1073 ± 185	1091 ± 84	927 ± 65

2.2 Experimental results for the optimization of LNA-DQAMmiR

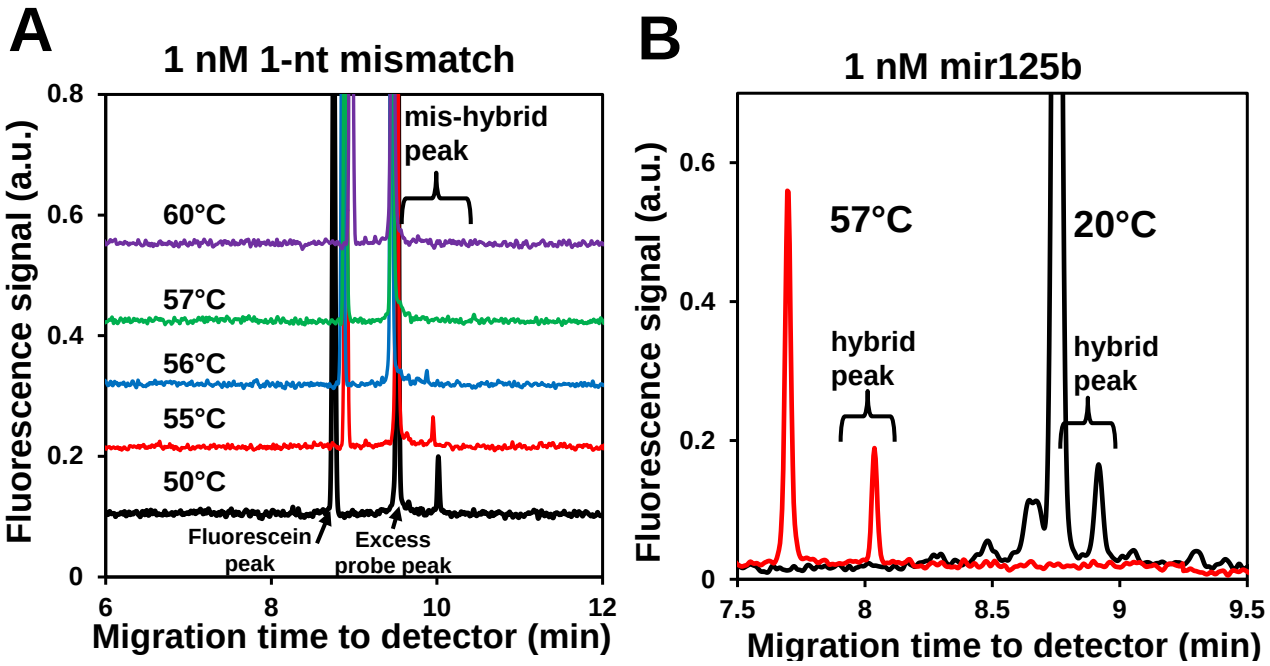

Figure S1. Optimization of capillary temperature to allow for single-nucleotide specificity. **Panel A:** 1 nM of a single-nucleotide mismatch of mir125b was incubated with 10 nM mir125b-specific LNA-DNA probe and injected into the capillary at varying temperatures. The mismatch hybrid peak was present at temperatures below 57°C, indicating that no mismatch binding occurs at 57°C or higher.
Panel B: 1 nM of mir125b was incubated with 10 nM mir125b-specific LNA–DNA probe and injected into the capillary at 57°C (red trace) and 1 nM of mir125b was incubated with 100 nM mir125b-specific LNA–DNA probe and injected into the capillary at 20°C (black trace) to determine if complete hybridization still occurred at 57°C. The 57°C hybrid peak was comparable to the 20°C hybrid peak indicating that complete hybridization occurs at 57°C. This showed that a capillary temperature of 57°C can achieve single-nucleotide specificity.

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:

$$[\text{miRNA}]^i = a\left(A_H^{\,i} / q_H^{\,i}\right) \tag{S3-1}$$

The known concentration of the *j*-th probe, $[P]_{0,j}$ can be expressed through the areas of two peaks, the one of SSB-bound excess probe, $A_{P,j}$, and the one of the miRNA-bound probe, $A_{H,j}$, with the same coefficient a and known quantum yields $q_{H,j}$ and $q_{P,j}$:

$$[P]_{0,j} = aA_{P,j} / q_{P,j} + A_{H,j} / q_{H,j} \tag{S3-2}$$

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

$$\sum_{j=1}^{N}[P]_{0,j} = a\left(\sum_{j=1}^{N} A_{P,j} / q_{P,j}\right) + a\left(\sum_{j=1}^{N} A_{H,j} / q_{H,j}\right) \tag{S3-3}$$

Since the peaks of the hybrids are resolved, their corresponding areas $A_{H,j}$ 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, $A_{P,j}$, as unknowns along with the coefficient a. While the individual $A_{P,j}$ 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 $A_{P,j}$ from $q_{P,j}$ by multiplying Equation S3-3 by $q_{P,j}$:

$$\sum_{j=1}^{N} q_{P,j}[P]_{0,j} = a\left(\sum_{j=1}^{N} A_{P,j}\right) + a\left(\sum_{j=1}^{N}\left(q_{P,j} / q_{H,j}\right) A_{H,j}\right) \tag{S3-4}$$

Equation S3-4 can be otherwise represented as:

$$\sum_{j=1}^{N} q_{P,j}[P]_{0,j} = aA_P + a\left(\sum_{j=1}^{N}\left(q_{P,j} / q_{H,j}\right) A_{H,j}\right) \tag{S3-5}$$

Now we can solve Equation S3-5 for a:

$$a = \frac{\sum_{j=1}^{N} q_{P,j}[P]_{0,j}}{A_P + \sum_{j=1}^{N}\left(q_{P,j} / q_{H,j}\right) A_{H,j}} \tag{S3-6}$$

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

$$\frac{[\mathrm{miRNA}]^{i} q_{\mathrm{H}}{}^{i}}{A_{\mathrm{H}}{}^{i}} = \frac{\sum_{j=1}^{N} q_{\mathrm{P},j}[\mathrm{P}]_{0,j}}{A_{\mathrm{P}} + \sum_{j=1}^{N}\left(q_{\mathrm{P},j} / q_{\mathrm{H},j}\right) A_{\mathrm{H},j}} \tag{S3-7}$$

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

$$[\mathrm{miRNA}]_{i} = \frac{A_{\mathrm{H},i}}{A_{\mathrm{P}} + \sum_{j=1}^{N}\left(q_{\mathrm{P},j} / q_{\mathrm{H},j}\right) A_{\mathrm{H},j}} \left(\sum_{j=1}^{N}\left(q_{\mathrm{P},j} / q_{\mathrm{H},i}\right)[\mathrm{P}]_{0,j} \right) \tag{S3-8}$$