Biosensors

DOI: 10.1002/anie.201001712

Multiplexed Detection and Label-Free Quantitation of MicroRNAs Using Arrays of Silicon Photonic Microring Resonators**

*Abraham J. Qavi and Ryan C. Bailey**

MicroRNAs (miRNAs) are short (19 to 24 nucleotides), single-stranded, non-protein-coding RNAs that are powerful transcriptional and post-transcriptional regulators of gene expression. Unlike small interfering RNAs (siRNAs), miRNAs are genomically encoded and play key roles in a range of normal cellular processes, including proliferation, apoptosis, and development.[1–4] Not surprisingly, miRNAs have also been implicated in a number of diseases, including cancer,[5–8] neurodegenerative disorders,[9–11] and diabetes,[12–14] and represent promising biomarker candidates for informative diagnostics. Despite their increasingly well-understood importance in gene regulation, the development of sensitive analytical techniques for the quantitation of multiple miRNAs has lagged behind. Furthermore, current methodologies for the analysis of miRNA expression are not applicable to a clinical setting where sample sizes are limited and assay cost and time-to-result is of tremendous importance.

In contrast to most technologies for nucleic acid analysis that advantageously utilize the polymerase chain reaction (PCR) to increase the amount of the target sequence, miRNAs are not easily amplified on account of their small size, which prohibits standard primer hybridization.[15] Although creative approaches that enable reverse transcriptase/PCR amplification have been developed,[16–18] many conventional miRNA analyses are prone to sequence-biased amplification or hindered by the need for large amounts of sample. The most widely reported technique for miRNA analysis technique, Northern blotting, requires substantial amounts of starting material, is extremely laborious, and is not amenable to large-scale multiplexing.[19] Recently, a number of new methods for miRNA analysis have been reported that feature high sensitivity, but often at the expense of assay simplicity and scalability, multiplexing capability, or rapid analysis time.[20–27]

We report herein a label-free, direct hybridization assay enabling the simultaneous detection of multiple different miRNAs from a single sample using commercially fabricated and modularly multiplexable arrays of silicon photonic microring resonators. Using single-stranded DNA capture probes, we are able to rapidly (10 min) quantitate down to approximately 150 fmol of miRNA and are able to discriminate between single nucleotide polymorphisms within the biologically important let-7 family of miRNAs. We also demonstrate the applicability of this platform for quantitative, multiplexed expression profiling by determining the concentration of four miRNAs from within a clinically relevant sample size of a cell line model of glioblastoma with minimal sample preparation.

Microring resonators are a promising class of refractive-index-sensitive devices that have recently been applied to monitoring chemical reactions and biomolecular binding events.[28–36] Light coupled by means of an adjacent linear waveguide is strongly localized around the circumference of the microring under conditions of optical resonance, as defined by the cavity geometry and the surrounding refractive-index environment. Given a defined microring structure, the resonance wavelength is sensitive to changes in the local refractive index, in this case the hybridization of miRNAs to complementary ssDNAs on the surface, as illustrated in Figure 1 a. By monitoring the shift in resonance wavelength after exposure to the sample of interest, the solution-phase analyte concentration can be determined.

We have previously described the use of silicon-on-insulator (SOI) microring resonators for the sensitive detection of proteins.[28–30] A wavelength-tunable laser centered at 1560 nm is coupled into on-chip waveguides that interrogate the microrings and determine resonance wavelengths. The sensor chips, each containing 32 individually addressable microrings 30 μm in diameter, are coated with a fluoropolymer cladding layer that is selectively removed over the active sensing elements using reactive ion etching. Figure 1 b shows a small portion of the sensor array, and the inset highlights a single microring and its adjacent linear interrogation waveguide.

The first step in modifying sensors to detect particular miRNAs is to covalently modify the native oxide-coated surface of the silicon microrings with single-stranded DNAs complementary to the target(s) of interest. After appropriate derivitization, the shifts in resonance wavelength accompanying hybridization of miRNA to the microrings can be followed in real time, as shown in Figure 2. At $t = 15$ minutes, a 2 μM solution of miR-24-1 is flowed over the sensors and its

[*] A. J. Qavi, Prof. Dr. R. C. Bailey
Department of Chemistry, Institute for Genomic Biology
and Micro and Nanotechnology Laboratory
University of Illinois at Urbana-Champaign
600 South Mathews Avenue, Urbana IL 61801 (USA)
Fax: (+1) 217-265-6290
E-mail: baileyrc@illinois.edu
Homepage: http://www.chemistry.illinois.edu/faculty/Ryan_Bailey.html

[**] This work was supported financially by the National Institutes of Health (NIH) Director's New Innovator Award Program, part of the NIH Roadmap for Medical Research, through grant number 1-DP2-OD002190-01, the Camille and Henry Dreyfus Foundation, and the Eastman Chemical Company (fellowship to A.J.Q.). We also thank Ji-Yeon Byeon for the SEM images of the microring resonator arrays.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201001712.

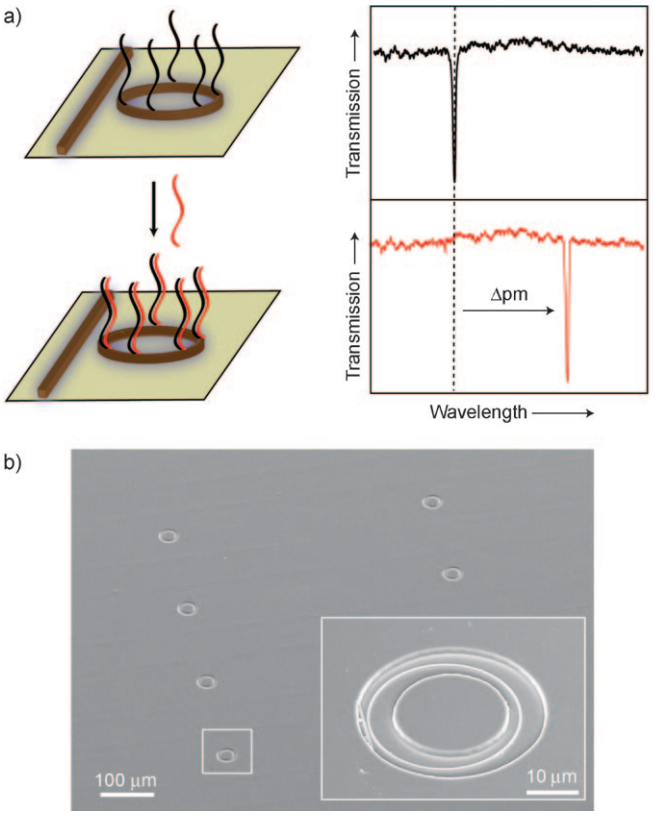

Figure 1. a) Each microring sensor is functionalized with a capture sequence of DNA (black). The sequence-specific hybridization of the target miRNA (red) causes a shift in the wavelength required to achieve optical resonance. b) Scanning electron micrograph showing six microrings on a sensor array chip. The inset shows a single microring and its corresponding linear access waveguide revealed within an annular opening in the fluoropolymer cladding layer.

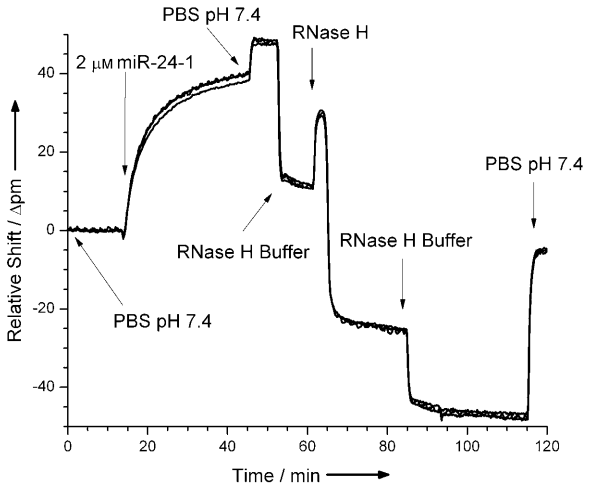

Figure 2. Real-time measurement of the shift of microring resonance wavelength during the hybridization of 2 μM miR-24-1 to three separate microring resonators. The resulting heteroduplex is subsequently dissociated by the enzyme RNase H, yielding a regenerated sensor surface.

hybridization to complementarily functionalized microrings elicits a shift of approximately 40 pm in the resonance wavelength. Returning to phosphate-buffered saline (PBS) buffer at $t = 45$ minutes gives an immediate increase in resonance peak shift on account of differences in the refractive index of the bulk solution. The opposite shift (a negative change in bulk refractive index) occurs for the injection of miRNA solution, but is largely counteracted by the hybridization of miRNA.

To confirm the hybridization, we introduced a solution containing RNase H, an enzyme that selectively cleaves DNA:RNA heteroduplexes, at $t = 60$ minutes. The rapid increase in resonance wavelength corresponds to a change in the bulk refractive index, but the enzymatic activity of RNase H dissociating the duplex quickly leads to a decrease in the relative peak shift. Control experiments without hybridized miRNA or with DNA:DNA duplexes show a stepped response that reflects only the bulk index change to and from the solution containing RNase H, but without the net decrease corresponding to heteroduplex cleavage. Returning the microring to RNase H buffer and then PBS buffer confirms the hybridization of miRNAs to the ssDNA capture strands and also demonstrates that the sensor surfaces can be regenerated. Utilizing this RNase H protocol, we have found that sensors can reproducibly respond to miRNA hybridization after more than twenty regeneration cycles.

Exposure of microrings to different solutions of miR-24-1 varying from 2 μM to 1.95 nM reveals a concentration-dependent response (Figure 3a). Rather than utilize the absolute wavelength shift, which saturates as miRNAs hybridize to all of the available ssDNA capture probes, we determine the rate at which the resonance peak changes immediately after target introduction and use the initial slope response for quantitation. Advantages of this approach include generation of a linear sensor calibration curve and greatly reduced assay time (roughly 10 min), which is significantly faster than waiting for the system to establish binding equilibrium, a concentration-dependent period that can take many hours. Figure 3b shows the linear relationship between the initial slope of sensor response, determined by fitting the real-time shift of the resonance wavelength, and the concentration of miR-24-1.

A significant challenge for all nucleic acid analyses that is particularly important for miRNAs is the ability to distinguish single-base differences in sequence. Therefore, we developed an isothermal method of distinguishing single-base differences between two members of the biologically important let-7 family of miRNAs by performing hybridizations in the presence of formamide, which is a chaotropic agent that competes for hydrogen-bonding sites. Under normal hybridization conditions (no formamide) the miRNA isoforms let-7b and let-7c, which differ only by a single-base change at position 17, both bind to the nonspecific DNA capture probe designed to be perfectly complementary to the other sequence (see Figure S6 in the Supporting Information). However, when hybridization is performed in a 50% (v/v) formamide solution, the single-base difference is easily distinguished.

A key advantage of the microring resonator sensing platform is its potential for high-level multiplexing. SOI

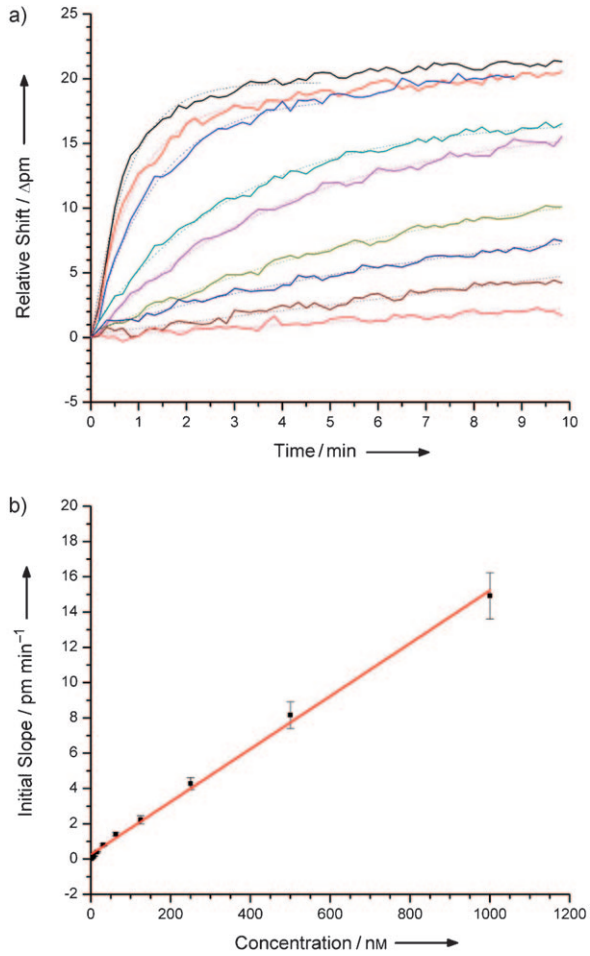

Figure 3. a) Response of a single microring to the binding of miR-24-1 as a function of concentration (2 μM to 7.8 nM decreasing top-to-bottom by twofold dilutions). The dotted lines designate the fitted curves used to calculate the initial slope of the target miRNA binding. The response of only a single ring is shown for clarity. Responses for miRNA concentrations of 3.91 and 1.95 nM are omitted for clarity, but are resolvable from the zero-concentration response. b) Average response of microring resonators as a function of miR-24-1 concentration. Error bars represent ±1 standard deviation for at least nine independent measurements at each concentration.

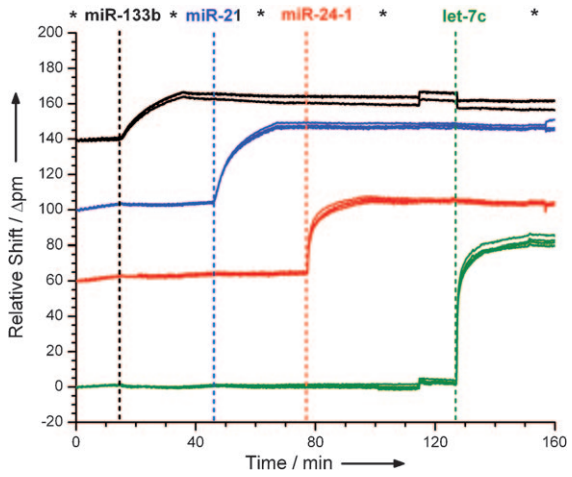

Figure 4. Sequence-specific detection of four unique miRNAs on a single chip as the miRNA complementary to the ssDNA on the microring is sequentially introduced into the flow chamber. Microrings were functionalized with complementary ssDNAs against (top to bottom) miR-133b, miR-21, miR-24-1, and let-7c. miRNA solutions were all 1 μM in PBS. Asterisks (*) denote time points at which the solution over the sensors was changed to PBS buffer. In some cases, small changes in resonance wavelength are observed resulting from small differences in bulk solution refractive index. Each set of rings is offset from the baseline wavelength for clarity.

microring resonators are fabricated using scalable semiconductor-processing techniques that enable a large number of sensors to be incorporated and individually interrogated on the same chip. Utilizing microarray spotting or other patterning methodologies, each ring can be functionalized with unique capture agents (cDNAs, antibodies, etc.), allowing many different biomolecules to be quantitated simultaneously.

To demonstrate the multiplexing capability of our platform, we constructed a four-component array by differentially functionalizing microrings on the same chip with unique ssDNAs complementary to four dissimilar miRNAs. Figure 4 shows the real-time shift in resonance wavelength for four sets of microrings, each functionalized with a different ssDNA, during the sequential introduction of miR-133b, miR-21, miR-24-1, and let-7c. Sequence-specific responses are observed at appropriate microrings only when the complementary miRNA solution is exposed to the sensor array. Small changes in resonance wavelengths arising from differences in bulk refractive index are observed at time points where solutions are switched, but in each case the sequence-specific response is clearly discernable above baseline.

Furthermore, we simultaneously determined the expression levels of the same four miRNAs extracted from U87 MG cells, an established model for grade IV gliomas, including glioblastoma and astrocytoma.[37,38] The entire small RNA content from 5×10^7 U87 cells was extracted using a commercial purification kit and flowed over a sensor surface with microrings functionalized with ssDNA capture probes complementary to the target miRNAs. Each microring was individually calibrated to account for differences in signal response between target miRNAs (see Figure S9 in the Supporting Information). The initial slope of sensor response upon addition of the small-RNA sample from U87 cells was measured and the concentration of each target miRNA in solution determined (miR-21: (18.9 ± 3) nM, miR-24-1: (3.3 ± 0.2) nM, miR-133b: (60 ± 20) nM, let-7c: (4 ± 3) nM).

Given the drive towards even smaller sample sizes, future work with this platform will focus heavily on improvements in sensitivity. One method for improving might include the incorporation of higher-affinity oligomer capture probes, such as locked nucleic acids (LNAs) and peptide nucleic acids (PNAs). Previous studies have shown that both classes of synthetic oligomers increase the specificity as well as sensitivity of miRNA assays.[26,39] Another approach might include the implementation of sequence-independent, secondary amplification techniques to increase the total mass bound to our sensor surfaces. Two candidate methods include the RNA-primed array-based Klenow enzyme assay (RAKE)

and Poly(A) polymerase enzymatic amplification, both of which utilize enzymes to specifically add nucleotides to the 3′ end of miRNAs hybridized to the sensor surface, after which additional amplification steps can be included to further boost the amount of bound mass.[21,40]

The emergence of miRNAs as important regulators of gene expression and as valuable disease biomarkers gives impetus on developing next-generation detection methodologies. Particularly valuable will be those that can operate under the sample-size limitations and time-to-result requirements of clinical analyses. Furthermore, multiplexed analyses in which a significant fraction of the "miRNA-ome", predicted to comprise roughly 1000 miRNAs for humans,[41] can be simultaneously analyzed will prove exceedingly important in deciphering the complex regulatory action of these molecules. In pursuit of these needs, we have developed a new platform for the sensitive, sequence-specific, and label-free quantitation of miRNAs using the direct hybridization to arrays of ssDNA-functionalized silicon photonic microring resonators. We demonstrate the ability to quantitate the expression level of multiple miRNAs from clinically relevant sample volumes within a data acquisition time of 10 minutes using a precalibrated sensor array. Future efforts will be directed towards improving sensor limits of detection as well as increasing levels of multiplexing by interfacing microring resonator arrays with microarray spotting technologies for the rapid encoding of many unique sensing elements.

Received: March 22, 2010
Published online: May 20, 2010

Keywords: analytical methods · biosensors · oligonucleotides · RNA

[1] R. C. Lee, R. L. Feinbaum, V. Ambros, *Cell* **1993**, *75*, 843.
[2] C. D. Johnson, A. Esquela-Kerscher, G. Stefani, N. Byrom, K. Kelnar, D. Ovcharenko, M. Wilson, X. W. Wang, J. Shelton, J. Shingara, L. Chin, D. Brown, F. J. Slack, *Cancer Res.* **2007**, *67*, 7713.
[3] M. Jovanovic, M. O. Hengartner, *Oncogene* **2006**, *25*, 6176.
[4] E. Wienholds, W. P. Kloosterman, E. Miska, E. Alvarez-Saavedra, E. Berezikov, E. de Bruijn, H. R. Horvitz, S. Kauppinen, R. H. A. Plasterk, *Science* **2005**, *309*, 310.
[5] P. T. Nelson, W.-X. Wang, B. W. Rajeev, *Brain Pathol.* **2008**, *18*, 130.
[6] J. Lu, G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebet, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz, T. R. Golub, *Nature* **2005**, *435*, 834.
[7] C. L. Bartels, G. J. Tsongalis, *Clin. Chem.* **2009**, *55*, 623.
[8] G. A. Calin, C. M. Croce, *Nat. Rev. Cancer* **2006**, *6*, 857.
[9] S. S. Hebert, B. De Strooper, *Science* **2007**, *317*, 1179.
[10] D. O. Perkins, C. D. Jeffries, L. F. Jarskog, J. M. Thomson, K. Woods, M. A. Newman, J. S. Parker, J. P. Jin, S. M. Hammond, *Genome Biol.* **2007**, *8*, R27.
[11] A. Schaefer, D. O'Carroll, C. L. Tan, D. Hillman, M. Sugimori, R. Llinas, P. Greengard, *J. Exp. Med.* **2007**, *204*, 1553.
[12] X. Tang, G. Tang, S. Özcan, *Biochim. Biophys. Acta Gene Regul. Mech.* **2008**, *1779*, 697.
[13] P. Muhonen, H. Holthofer, *Nephrol. Dial. Transplant.* **2009**, *24*, 1088.
[14] M. D. Walker, *Diabetes* **2008**, *57*, 2567.
[15] A. W. Wark, H. J. Lee, R. M. Corn, *Angew. Chem.* **2008**, *120*, 654; *Angew. Chem. Int. Ed.* **2008**, *47*, 644.
[16] J. Li, B. Yao, H. Huang, Z. Wang, C. Sun, Y. Fan, Q. Chang, S. Li, X. Wang, J. Xi, *Anal. Chem.* **2009**, *81*, 5446.
[17] C. K. Raymond, B. S. Roberts, P. Garrett-Engele, L. P. Lim, J. M. Johnson, *RNA* **2005**, *11*, 1737.
[18] C. F. Chen, D. A. Ridzon, A. J. Broomer, Z. H. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak, K. J. Guegler, *Nucleic Acids Res.* **2005**, *33*, e179.
[19] K. A. Cissell, S. Shrestha, S. K. Deo, *Anal. Chem.* **2007**, *79*, 4754.
[20] R.-Q. Liang, W. Li, Y. Li, C.-y. Tan, J.-X. Li, Y.-X. Jin, K.-C. Ruan, *Nucleic Acids Res.* **2005**, *33*, e17.
[21] S. Fang, H. J. Lee, A. W. Wark, R. M. Corn, *J. Am. Chem. Soc.* **2006**, *128*, 14044.
[22] S. P. Jonstrup, J. Koch, J. Kjems, *RNA* **2006**, *12*, 1747.
[23] J. Li, S. Schachermeyer, Y. Wang, Y. Yin, W. Zhong, *Anal. Chem.* **2009**, *81*, 9723.
[24] Y. Q. Cheng, X. Zhang, Z. P. Li, X. X. Jiao, Y. C. Wang, Y. L. Zhang, *Angew. Chem.* **2009**, *121*, 3318; *Angew. Chem. Int. Ed.* **2009**, *48*, 3268.
[25] H. Yang, A. Hui, G. Pampalakis, L. Soleymani, F.-F. Liu, E. H. Sargent, S. O. Kelley, *Angew. Chem.* **2009**, *121*, 8613; *Angew. Chem. Int. Ed.* **2009**, *48*, 8461.
[26] G. J. Zhang, J. H. Chua, R. E. Chee, A. Agarwal, S. M. Wong, *Biosens. Bioelectron.* **2009**, *24*, 2504.
[27] Y. Zhang, Z. Li, Y. Cheng, X. Lv, *Chem. Commun.* **2009**, 3172.
[28] A. L. Washburn, L. C. Gunn, R. C. Bailey, *Anal. Chem.* **2009**, *81*, 9499.
[29] A. L. Washburn, M. S. Luchansky, A. L. Bowman, R. C. Bailey, *Anal. Chem.* **2010**, *82*, 69.
[30] M. S. Luchansky, R. C. Bailey, *Anal. Chem.* **2010**, *82*, 1975.
[31] M. Iqbal, M. Gleeson, B. Spaugh, F. Tybor, W. G. Gunn, M. Hochberg, T. Baehr-Jones, R. C. Bailey, L. C. Gunn, *IEEE J. Sel. Top. Quantum Electron.* **2010**, DOI: 10.1109/JSTQE.2009.2032510.
[32] D. X. Xu, A. Densmore, A. Delage, P. Waldron, R. McKinnon, S. Janz, J. Lapointe, G. Lopinski, T. Mischki, E. Post, P. Cheben, J. H. Schmid, *Opt. Express* **2008**, *16*, 15137.
[33] C. Y. Chao, W. Fung, L. J. Guo, *IEEE J. Sel. Top. Quantum Electron.* **2006**, *12*, 134.
[34] A. Yalcin, K. C. Popat, J. C. Aldridge, T. A. Desai, J. Hryniewicz, N. Chbouki, B. E. Little, O. King, V. Van, S. Chu, D. Gill, M. Anthes-Washburn, M. S. Unlu, *IEEE J. Sel. Top. Quantum Electron.* **2006**, *12*, 148.
[35] K. De Vos, J. Girones, S. Popelka, E. Schacht, R. Baets, P. Bienstman, *Biosens. Bioelectron.* **2009**, *24*, 2528.
[36] A. Ramachandran, S. Wang, J. Clarke, S. J. Ja, D. Goad, L. Wald, E. M. Flood, E. Knobbe, J. V. Hryniewicz, S. T. Chu, D. Gill, W. Chen, O. King, B. E. Little, *Biosens. Bioelectron.* **2008**, *23*, 939.
[37] M. J. Clark, N. Homer, B. D. O'Connor, Z. G. Chen, A. Eskin, H. Lee, B. Merriman, S. F. Nelson, *PLoS Genet.* **2010**, *6*, e1000832.
[38] http://www.attc.org.
[39] A. Valoczi, C. Hornyik, N. Varga, J. Burgyan, S. Kauppinen, Z. Havelda, *Nucleic Acids Res.* **2004**, *32*, e175.
[40] P. T. Nelson, D. A. Baldwin, L. M. Scearce, J. C. Oberholtzer, J. W. Tobias, Z. Mourelatos, *Nat. Methods* **2004**, *1*, 155.
[41] I. Bentwich, A. Avniel, Y. Karov, R. Aharonov, S. Gilad, O. Barad, A. Barzilai, P. Einat, U. Einav, E. Meiri, E. Sharon, Y. Spector, Z. Bentwich, *Nat. Genet.* **2005**, *37*, 766.

