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Rolling-Circle Amplification: Unshared Advantages in miRNA Detection

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Micro RNAs (miRNA) are short regulatory RNA molecules of 21 nucleotides (nt) in length. In recent years, data on downstream effects of miRNA post-transcriptional gene regulation has been vastly accumulating. It has been shown that miRNAs play an important role in cell regulation, differentiation and disease formation.[1] Comparative miRNA profiling has revealed several miRNAs as potential drug targets and intensive work is currently being invested in drug design.[2] In order to promote further mechanistic studies and future diagnostic applications reliable quantification of specific miRNA from total cellular RNA is essential. Because miRNAs are of exceptionally short length, have very similar nucleotide sequences and often have very low expression levels, they set new demands on current miRNA profiling techniques.[3] Recently in *Angewandte Chemie*, Cheng et al. presented a new facile method for specific and sensitive miRNA quantification.[4] Undoubtedly, this novel tool will significantly contribute to future advances in research towards miRNA mechanism of action, its biological role and applications in clinical diagnostics.

Many details of the miRNA mechanism of action are still subject to intensive investigation. miRNAs are transcribed from miRNA genes as long primary miRNAs (pri-miRNAs) that undergo several maturation steps. In the cell nucleus pri-miRNAs are processed by the RNase III enzyme Drosha to approximately 75 nt hairpin precursor miRNAs (pre-miRNAs). After transportation into the cytoplasm by exportin-5 the hairpin is nucleolytically cleaved to approximately 22 nt long miRNA duplexes. Of this duplex the mature leading miRNA is recruited to the RNA-induced silencing complex (RISC), which then sequence specifically binds to its target mRNA. This leads to either translational repression, mRNA degradation or in some cases translational activation.[5] Until very recently, mRNA degradation was thought to be reserved only to fully complementary miRNA. However, the decay of mRNA has now advanced to the most probable general mechanism for gene down-regulation through miRNAs.[6] Another recent finding reinvents the role of the passenger strand (miRNA*). While in the past only the mature leading miRNA was known to be functionally active, several studies have shown that also the passenger miRNA* exhibits function at low but significant levels, and has target sequences within the 3′ untranslated region (3′UTR) of mRNA.[7] This not only hints at the complexity of this regulatory machinery, but at the same time demonstrates the crucial need for improved sensitivity in miRNA detection methods.

The biological role of miRNAs is not yet fully understood. So far the number of known human mature miRNAs has increased to 695 with an estimate of about another 300 to be discovered.[8,9] It is believed that miRNAs regulate 30% of the human genome.[10] Expression levels of specific miRNAs can range from a few to several thousand copies per cell depending on cell type and developmental stage.[5] It is known that several miRNAs function as regulators of cell development, proliferation, metabolism and apoptosis. The comparison of miRNA profiles is not only used to unravel new regulatory functions, but is also a means to identify the roles of specific miRNAs in dysfunctional cells.

Many human diseases strongly correlate with altered miRNA expression levels; this suggests that miRNA quantification is necessary for a wide range of applications in clinical diagnostics. With the advancement of miRNA microarray profiling technology expression levels of several hundred miRNAs could be compared between normal and affected cells, and potential disease specific, regulating miRNAs identified. For this purpose, a number of clinical trials are ongoing or are recruiting patient samples.[11]

The need for mutation-specific miRNA profiling methods is emphasized by a recent biochemical study. Heterozygous individuals of a single nucleotide polymorphism in the precursor miR-146a display a greater risk of papillary thyroid carcinoma (PTC). Considering that passenger strand miRNAs* can exhibit regulatory functions, only heterozygous individuals produce three different mature miRNAs: the leading mir-146a and the passengers mir-146a*G and mir-146*C. Thus, these individuals regulate a greater pool of genes than homozygotes for either GG or CC. Having shown that these genes regulate biological processes, which are involved in tumorigenesis, the authors proposed a possible cause for the predisposition of heterozygotes to PTC.[12]

Current miRNA detection methods are often laborious, time-consuming and intricate.[3] Northern blotting remains the standard method but like single-molecule fluorescence or surface plasmon resonance methods, it is unsuitable for high-throughput (HT) formats. However, in HT applications, such as quantitative real-time PCR or microarrays, it is a challenge to remain specific and sensitive, since miRNAs constitute a very small percentage of total cellular RNA and cover a range of four magnitudes of copies per

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cell. Therefore, size purification of cellular RNA is inevitable and in cases in which expression levels are low, normally PCR amplification is necessary prior to quantitative detection. However, the short length of miRNAs even makes standard applications, such as PCR, demanding. Since the melting temperatures (T_m) of different miRNAs can vary within 30 °C, they must be normalized by shortening miRNA probes or primers.[13] Unfortunately, this leads to decreased specificity, which cannot be tolerated since miRNAs of the same family often only differ by a single nucleotide. LNA/DNA probes are now being employed in all methods involving hybridization to antagonize this trend.[13]

In their recent publication, Cheng et al. describe the highly sensitive and specific quantitative detection of miRNAs using rolling circle amplification (RCA).[4] RCA is well established for the specific and sensitive detection of short nucleotide sequences and was first applied to miRNAs by Jonstrup et al.[14] This technique employs the analyte miRNA as a primer for DNA polymerase driven amplification of a circular DNA. The two ends of a linear DNA—a so-called padlock probe—are designed to have adjacent complementary sequences on a targeted miRNA. In the presence of a hybridizing miRNA, the 3′-OH and 5′-PO_4 ends of the DNA probe sequence specifically come within close vicinity of each other and are normally ligated with T4 DNA ligase; this gives rise to circular DNA (Figure 1 A). The miRNA can now serve as a primer in an isothermal polymerase reaction with phi29 DNA polymerase and lead to linear amplification of the padlock probe, which can be detected by using gel electrophoresis (Figure 1 B). Cheng et al. were able to significantly improve both specificity and sensitivity with minimal but efficient tools. Their key to greater specificity was the usage of a different ligase and temperature optimization of the ligation reaction. Whereas T4 DNA ligase has been reported to merge single stranded 3′-OH and 5′-PO_4 ends even in the absence of a template, which results in high background reactions or even false positive RCA,[15] T4 RNA ligase 2 (RnL2) is strictly dependent on duplex RNA or RNA–DNA hybrids. In addition, this enzyme is very sensitive towards 1 nt gaps and flaps, which establishes ideal prerequisites for miRNA-dependent RCA.[16] Although RnL2 has optimal activity at 37 °C, increase in temperature towards the T_m of the specific miRNA led to further enhancement of specificity. Regarding sensitivity, Cheng et al. again applied two simple, but effective modifications. Instead of performing linear amplification, they decided to introduce a second primer; this resulted in exponential "branched" amplification of the padlock probe (Figure 1 C).[17] Quantification was achieved by using homogeneous detection with SYBR-Green, which circumvented time-consuming gel electrophoresis. In order to demonstrate the power of their method, the authors set up a realistic case study with the biologically relevant let-7 miRNA family, which regulate three major oncogenes RAS, MYC and HMGA2. Very low expression levels of let-7 could be correlated to patients with advanced lung cancers, which makes let-7 a potential diagnostic marker.[18] Cheng et al. chose three miRNAs of the let-7 family bearing perfect complementarity, one- or two-nucleotide mismatches to the padlock probe. In terms of specificity they could achieve sevenfold discrimination between full complementarity and 1 nt mismatch, located 3 nt from the ligation site. A linear dependence between probe quantity and fluorescence intensity was established in the range of 0.025 to 1 pM, though the detection limit is reportedly about 10 fM. Cheng et al. present a simple and robust method for miRNA quantification with state-of-the-art specificity and sensitivity. This straightforward and low-tech method completely relies on commercially available chemicals and enzymes, and is not dependent on sophisticated equipment. Thus, it can be expected that this method will quickly find broad application in many laboratories involved in miRNA research.

Although in principle this method allows extension to multiplex miRNA analysis, as with its competitors, it will have similar challenges with specificity in coherence with T_m values of different miRNAs. However, it can be expected that RnL2 will at least to some extent prevent ligation on erroneously hybridized templates. Furthermore, in some cases it can be assumed that pre-miRNAs could also give rise to DNA polymerization; this would make prior size-purification of total cellular RNA essential.

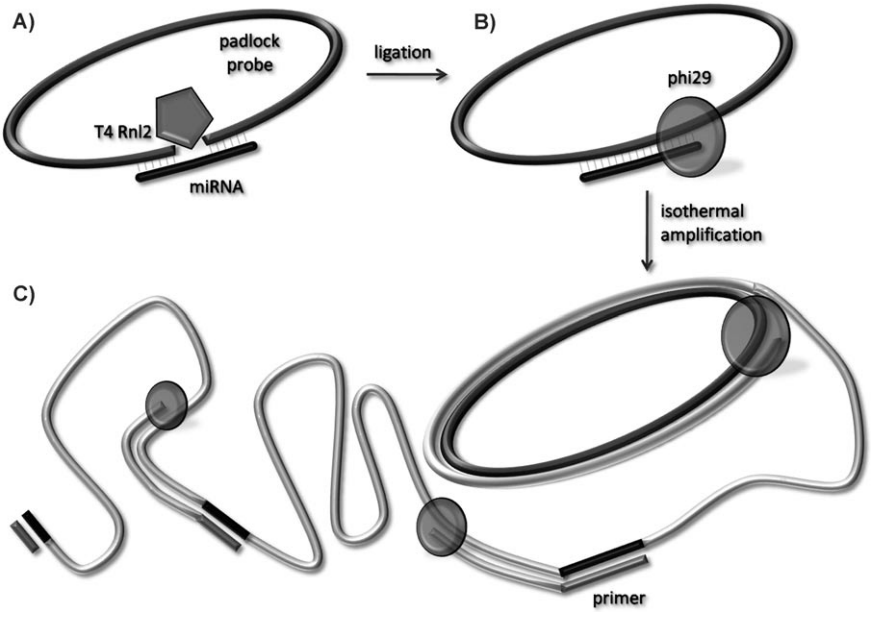

Figure 1. A) Specific miRNA-templated ligation of the linear padlock probe with RNA ligase 2. B) Phi29 DNA polymerase uses hybridized miRNA as primer. C) Isothermal displacing polymerization with circular padlock probe as template. Secondary primers act as branching points for exponential synthesis of DNA.

The inherent nature of miRNAs makes quantitative detection and profiling methods complex, but they exhibit vital postregulatory functions in the cell. Dysregulation of miRNA function is a cause and indicator for disease and disease progression. Determination of miRNA expression levels could allow the diagnosis of complex diseases, such as breast cancer, Parkinson's, and Alzheimer's, in their early asymptomatic stages. Especially the diagnosis of cancers and assignment of cells to a primary tumor or to metastases is often time-consuming and error-prone. Indeed, metastatic cancer of unknown primary site (CUP) is one of the ten most frequent cancer diagnoses in humans.[19] In an early but impressive study, it could be shown that a set of 217 miRNA expression levels allowed a correct classification (12 out of 17 cases) of poorly differentiated tumors.[20] Interestingly, a set of 16000 mRNA expression levels could only correctly assign one out of 17 tumors that could not be analyzed with standard histopathology methods. A rapid and reliable diagnosis, however, is indispensable for the treatment of human disease. Rolling circle amplification as performed by Cheng et al. represents a benchmark for simple, specific and sensitive quantification of miRNAs, and might play an important role in the future progress of this fascinating field of research.

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[1] G. Stefani, F. J. Slack, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 219–230.
[2] C. Arenz, *Angew. Chem.* **2006**, *118*, 5170–5172; *Angew. Chem. Int. Ed.* **2006**, *45*, 5048–5050.
[3] A. W. Wark, H. J. Lee, R. M. Corn, *Angew. Chem.* **2008**, *120*, 654–663; *Angew. Chem. Int. Ed.* **2008**, *47*, 644–652.
[4] Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang, Y. Zhang, *Angew. Chem. Int. Ed.* **2009**, DOI 10.1002/anie.200805665.
[5] D. P. Bartel, *Cell* **2004**, *116*, 281–297.
[6] S. Esslinger, K. Förstemann, *Angew. Chem.* **2009**, *121*, 867–869; *Angew. Chem. Int. Ed.* **2009**, *48*, 853–855.
[7] K. Okamura, M. D. Phillips, D. M. Tyler, H. Duan, Y. T. Chou, E. C. Lai, *Nat. Struct. Mol. Biol.* **2008**, *15*, 354–363.
[8] S. Griffiths-Jones, H. K. Saini, S. van Dongen, A. J. Enright, *Nucleic Acids Res.* **2008**, *36*, D154–D158.
[9] E. Berezikov, V. Guryev, J. van de Belt, E. Wienholds, R. H. Plasterk, E. Cuppen, *Cell* **2005**, *120*, 21–24.
[10] B. P. Lewis, C. B. Burge, D. P. Bartel, *Cell* **2005**, *120*, 15–20.
[11] For more detail see: http://www.clinicaltrials.gov/ct2/results?term=miRNA
[12] K. Jazdzewski, S. Liyanarachchi, M. Swierniak, J. Pachucki, M. D. Ringel, B. Jarzab, A. de La Chapelle, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1502–1505.
[13] W. Li, K. Ruan, *Anal. Bioanal. Chem.* **2009**, DOI 10.1007/s00216-008-2570-2.
[14] S. P. Jonstrup, J. Koch, J. Kjems, *RNA* **2006**, *12*, 1747–1752.
[15] H. Kuhn, M. D. Frank-Kamenetskii, *FEBS J.* **2005**, *272*, 5991–6000.
[16] J. Nandakumar, C. K. Ho, C. D. Lima, S. Shuman, *J. Biol. Chem.* **2004**, *279*, 31337–31347.
[17] P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, D. C. Ward, *Nat. Genet.* **1998**, *19*, 225–232.
[18] I. Büssing, F. J. Slack, H. Grosshans, *Trends Mol. Med.* **2008**, *14*, 400–409.
[19] N. Pavlidis, E. Briasoulis, J. Hainsworth, F. A. Greco, *Eur. J. Cancer* **2003**, *39*, 1990–2005.
[20] J. Lu, G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz, T. R. Golub, *Nature* **2005**, *435*, 834–838.

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