New Application for the LightCycler® 480 System: qPCR-based microRNA-Profiling

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Recent studies indicate that microRNAs (miRNAs) are mechanistically involved in the development of various human malignancies and therefore represent a promising new class of biomarkers. Quantitative PCR-based technologies are emerging as the gold standard for miRNA profiling. Here, a commercially available miRNA assay was used to measure and compare the expression levels of 154 miRNAs of seven lymph node samples using the LightCycler® 480 Instrument and a competitor real-time PCR system. We observed highly similar results for 134 miRNAs (87%) and some larger deviations (20 miRNAs/13%). In addition to large-scale miRNA profiling, it is also possible to use the LightCycler® 480 System in the assessment of miRNA expression in small sample sizes. We have conclusively demonstrated that miR-205 can be detected in a dose-dependent manner in a dilution series of 50, 10, 5, and 1 cell(s) of the KU19-19 cell line. This result highlights one of the major advantages of qPCR-based miRNA profiling over array-based technologies – highest sensitivity and log-linear amplification over at least six orders of magnitude.

Introduction

The human genome is predicted to code for approximately 1,000 miRNAs, 474 of which are currently listed as experimentally validated miRNAs in the Sanger database (www.microrna.sanger.ac.uk/sequences). The miRNAs are transcribed from RNA genes to result in primary miRNA (pri-miRNA/several hundred basepairs), which are subsequently processed to result first in hairpin-like pre-miRNAs (~90 bp) and finally in mature miRNAs (~22 bp) [1]. In mammals, most miRNAs specifically bind to an imperfectly matching complementary sequence in the 3´-untranslated region (3´-UTR) of a target gene, thereby inhibiting translation [1]. On average, each individual miRNA is predicted to target approximately 200 target genes [2]. The 3´-UTR of each mRNA offers a strongly varying number of different miRNA target sites, ranging from 0 to >30. In summary, the translation of more than 30% of all genes may be directly regulated by miRNAs [2]. Signal cascades involved in processes such as differentiation and proliferation are predominately targeted by miRNAs [3]. Because tumor cells are usually characterized by dedifferentiation and enhanced proliferation, it appears easy to envision differential expression of individual miRNAs as a potential cause of tumor development.

Calin *et al.* were the first to describe a common downregulation of miR-16/15a in chronic lymphocytic leukemias (CLL) and subsequently demonstrated that a specific miRNA signature was associated with prognostic factors and progression of the disease [4,5]. Thereafter, an increasing number of publications analyzed the involvement of miRNA expression in cancer development. This process was accelerated by the advent of miRNA profiling (May 2007: 271 publications using the keywords 'miRNA' and 'cancer'). The first organized approach to measuring a large number of miRNAs was published by Lu *et al.* [6]. A FACS-based method was used to profile 217 miRNAs from 334 human tissues, including various tumor samples. It was demonstrated that each tissue provides a specific miRNA signature, similar to – and at least as informative as – gene expression signatures. Evidence was provided for the conclusion that miRNA profiles not only describe the current state of cell differentiation, but also reflect the developmental origin of the tissue.

To date, a potpourri of methods have been invented to profile miRNA expression. While miRNA microarrays have been most broadly applied, there are concerns, because

Table 1: Overall signal intensities (Cp) of 7 lymph node miRNA profiles (154 miRNAs) are depicted. Highly similar results were obtained for both real-time PCR instruments as deduced from small ΔCp values.

	LightCycler® 480 Instrument	Competitor system	ΔCp
Lymph node 1	35.5	35.8	-0.3
Lymph node 2	36.5	36.3	0.2
Lymph node 3	36.2	35.5	0.7
Lymph node 4	35.6	35.7	-0.1
Lymph node 5	35.9	36.3	-0.4
Lymph node 6	36.8	36.4	0.4
Lymph node 7	36.2	35.8	0.4

this method is likely to detect miRNA precursors as well as mature miRNAs. Furthermore, miRNA microarrays are not suitable for discriminating between miRNA species of high similarity and can lead to false-positive results. Here, the LightCycler® 480 System is used as an instrument to carry out an extremely sensitive and highly specific qPCR-based method of miRNA profiling.

Materials and Methods

Total RNA was extracted using the RecoverAll kit (Ambion, Austin, Texas, USA). miRNA expression profiling was performed according to the TaqMan® MicroRNA Assay protocol (PE Applied Biosystems, Foster City, CA) and as described by Bandres *et al.* [7]. The qPCR reactions (10 µl) for the cell dilution experiments contained: 5 µl LightCycler® 480 Probes Master, 3.33 µl nuclease-free water, 0.67 µl miRNA-cDNA first strand, 1 µl primer (included in the microRNA Assay Kit). The following program was used on the LightCycler® 480 Instrument: Enzyme activation: 95°C for 10 minutes; amplification (45 cycles): 95°C for 15 seconds (ramp: 4,4°C/s, analysis mode: quantification), 60°C for 60 seconds (ramp: 2,2°C/second); cooling: 40°C for 30 seconds (ramp: 2°C/second). The detection format was set to 'Mono Color Hydrolysis Probe' and the second derivative maximum method was used for absolute quantification.

Results and Discussion

In our group we started miRNA profiling in October 2005, using the real-time PCR machine of another group on campus. Expression profiling of 154 miRNAs was commenced on samples of diffuse large B-cell lymphoma. The LightCycler® 480 System was introduced in Germany in September 2005.

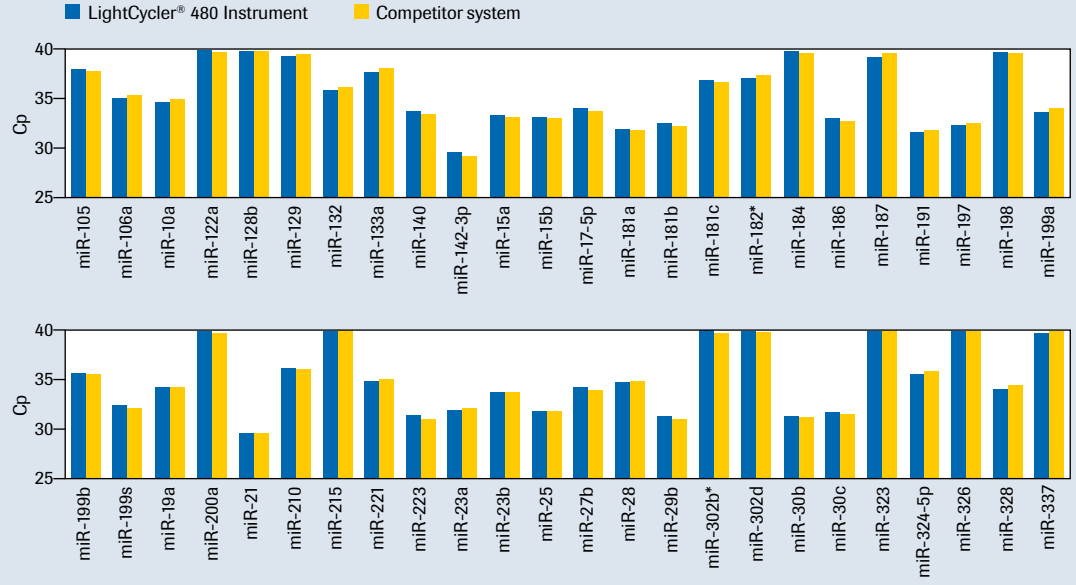

Figure 1: Average Cp values from 7 lymph node miRNA profiles are shown for a selection of 48 miRNAs. Expression results obtained from the LightCycler® 480 System are similar to those measured by the competitor system.

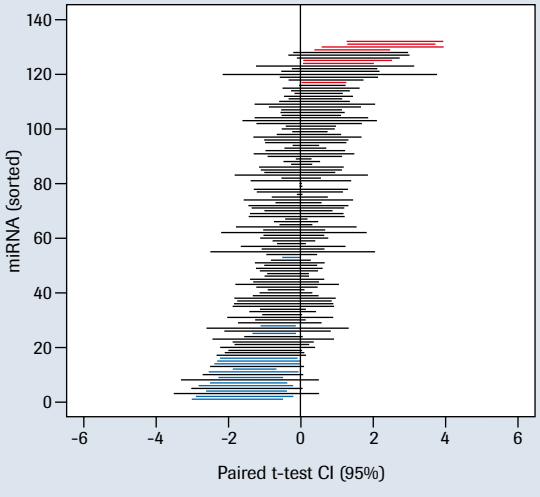

Figure 2: Confidence intervals (95%) for the paired t-test comparing the two PCR techniques, sorted according to estimated difference. Most confidence intervals include the null hypothesis value of zero difference.

At this point we started to realize the advantages of this device over the one we were then using. These advantages included: easier data transfer to Excel, easier block exchange, less waste heat, smaller footprint, and a much lower price. Therefore, we were easily convinced to perform test runs to compare miRNA-profiling results of the LightCycler® 480 Instrument with those of the competitor system.

Total RNA was isolated from formalin-fixed paraffin-embedded tissue of 7 lymph nodes. miRNA profiling was then carried out in two steps: 1. individual cDNA first-strand synthesis for each of the 154 miRNAs; 2. qPCR reactions. 7 complete lymph node miRNA-cDNA sets served as template to measure 7 miRNA profiles using the LightCycler® 480 Instrument and a further 7 profiles using the competitor system. In total, 2,156 qPCR reactions resulted in two sets of 1,078 Cp values. Using

default settings on both devices the overall signal intensity was determined by calculating the average Cp of each of the 2x7 miRNA profiles. The results obtained were highly similar (Table 1). The average Cp over all 7 miRNA profiles was 36.1 for the LightCycler® 480 Instrument and 36.0 for the competitor system. The overall standard deviation was 0.1 (Table 1). For more detail, Figure 1 shows the average expression intensity of 48 individual miRNAs, derived from seven lymph node samples, as determined by the LightCycler® 480 Instrument and the competitor system, respectively. These results clearly demonstrate that the biological variance between the analyzed samples as well as the technical variance (*e.g.*, pipetting errors, real-time device used for detection, algorithm used for the calculation of the Cp) is small. Therefore, the LightCycler® 480 Instrument is well-suited for qPCR-based miRNA profiling.

In addition, we were curious to find out whether it was possible to directly compare results for each individual miRNA obtained from both real-time machines. Therefore, confidence intervals (95%) were calculated for the paired t-test depicted in Figure 2. Most confidence intervals include the null hypothesis value of 0 difference, demonstrating that 84% of all miRNA expression results are not biased by either the detection device or the algorithms used for Cp calculation. We also tested whether the TaqMan® PCR master mix required for the quantification of miRNA expression could be replaced by the LightCycler® 480 Probes Master, by comparing the results of two complete lymph node miRNA profiles. The two data sets were nearly identical (data not shown). The LightCycler® 480 Probes Master was used for the experiments described below.

The analysis of cancer tissues is often accompanied by the need to analyze a limited number of cells, which in turn requires highly sensitive detection methods, such as quantitative PCR. Tissue-specific miRNAs are often expressed in extremely high copy numbers (*e.g.*, an estimated 66,000 copies of miR-122a per liver cell), benefiting expression analysis of very small tissue samples. Here we determined the expression intensity of miR-205 in a dilution series of KU-19-19 cells. It was known from previous experiments that miR-205 is abundantly expressed in this urinary bladder carcinoma cell line. Cells were diluted in PBS and the heat-disrupted lysate (10 minutes, 95°C) was directly used for miRNA-cDNA synthesis and qPCR. We were able to determine a dose-dependent miRNA expression in 50, 10, 5, and 1 cell(s), as depicted in Figure 3. The theoretical and the detected fold-change between dilution steps matched well. Similar results were obtained with laser capture microdissected and/or formalin-fixed paraffin-embedded tissue samples, measuring the ubiquitously expressed miR-16. These data demonstrate that expression studies of selected miRNAs are easily performed using very small tissue samples.

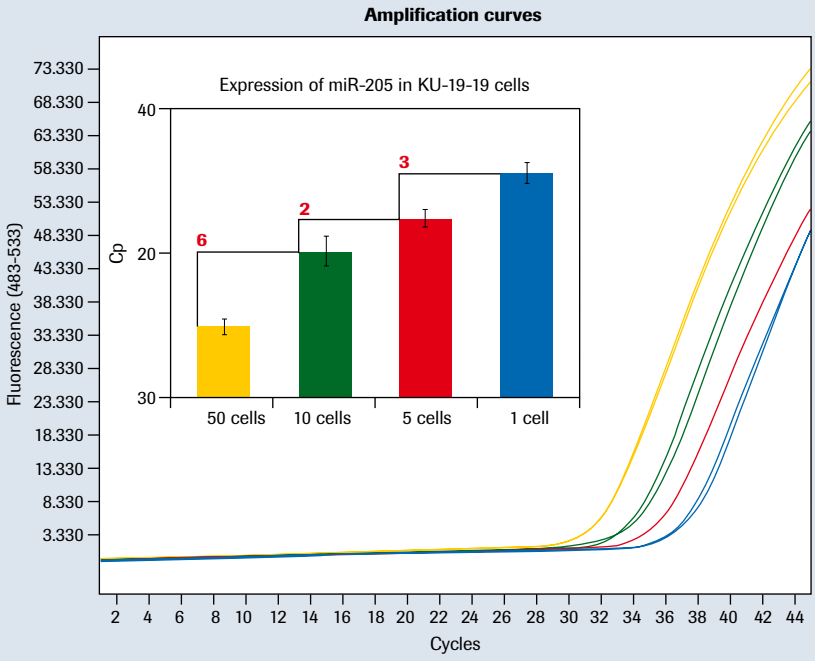

Figure 3: A dilution series (50, 10, 5, and 1) of urinary bladder carcinoma cells (KU-19-19) was used to quantify the expression of miR-205. Theoretical dilution factors were 5-fold, 2-fold and 5-fold and corresponded well with those determined by qPCR (6-fold, 2-fold and 3-fold/red numbers). Measurements were performed in duplicate. 50 cells (yellow), 10 cells (green), 5 cells (red), and 1 cell (blue).

Conclusions

The LightCycler® 480 System is our preference for large-scale miRNA profiling. An automated liquid handling system is now set-up to analyze 384 miRNAs in parallel. Furthermore, miRNA expression quantification as performed here on the LightCycler® 480 System is by far the most sensitive method available, superior over all array-based miRNA profiling systems. ■

References

1. He L, Hannon GJ (2004) Nat Rev Genet 5:522–531
2. Lewis BP *et al.* (2005) Cell 120:15–20
3. Hwang HW, Mendell JT (2006) Br J Cancer 94:776–780
4. Calin GA *et al.* (2002) Proc Natl Acad Sci USA 99:15524–15529
5. Calin GA *et al.* (2005) N Engl J Med 353:1793–1801
6. Lu J *et al.* (2005) Nature 435:834–838
7. Bandres E *et al.* (2006) Mol Cancer 5:29

Order INFO

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument	1 Instrument (96 well)	04 640 268 001
LightCycler® 480 Instrument	1 Instrument (384 well)	04 545 885 001
LightCycler® 480 Probes Master	5 x 1ml (2 x conc.)	04 707 494 001
LightCycler® 480 Probes Master	10 x 5ml (2 x conc.)	04 887 301 001