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Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers

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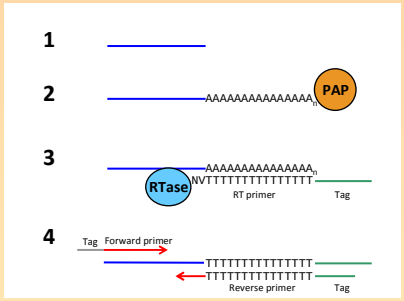
Abstract

MicroRNAs are important regulators of gene expression and it is of great interest to quantitatively determine their expression in different biological settings. Here, we describe a qPCR method for quantification of microRNAs based on a single reverse transcription reaction for all microRNAs combined with real-time PCR with two, microRNA-specific DNA primers.



The method is able to readily discriminate between microRNAs with single nucleotide differences. Importantly, qPCR with DNA primers yields significantly higher amplification efficiencies than a similar method based on locked nucleic acids-spiked primers. The higher amplification efficiency of DNA primers translates into higher sensitivity and precision in microRNA quantification.

Background

One of the most frequently used methods for detection of miRNAs is RT qPCR. Several methods have been developed to overcome the problem of the short length of the microRNAs:

* Chen and coworkers (2005) developed stem–loop RT–PCR. Unfortunately, this method does not allow the user to control the specificity of the reaction by melting curve analysis.

* Shi *et al.* (2005) developed the method of poly(A) tailing and use of a tagged poly(T) primer for reverse transcription. Only one microRNA specific primer is used in the qPCR which makes difficult to discriminate between closely related miRNAs.

* Kumar *et al.*(2010) have developed a method based on circularization of the miRNA which also depends on a specific primer for reverse transcription and gives difficulties in multiplexing.

* Busk (2010) developed the Universal RT microRNA PCR method which combines the benefits of a universal RT reaction with the specificity of two miRNA-specific qPCR primers spiked with LNA. These primers often exhibit a low amplification efficiency.

Materials & Methods

Total RNA preparation
-TRIzol® reagent (Invitrogen) or with TRI Reagent® (MRC).
-RNA quality was examined on Bioanalyzer (Agilent) or by visual inspection.

cDNA synthesis
- 100 ng of total RNA was used for cDNA synthesis essentially as described by Busk (2010).
-For the microRNA LNA™ PCR kit (Exiqon), cDNA synthesis was done according to the manufacturer's instructions.

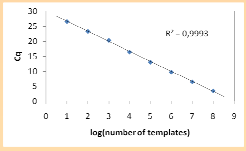
PCR primer Design
-All DNA PCR primers were designed as previously described by Busk (2010) but without LNA spikes.
-Tm was optimized to 59 °C by adjusting the tail length of the primers and calculated according to the nearest-neighbor model.
-An *in-house* program has been made to design primers to be used by this method. For primer design contact pkb@bio.aau.dk.

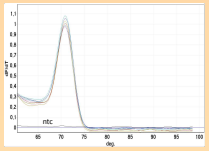
qPCR
-qPCR was done using QuantiFast SYBR Green PCR master mix (Qiagen) or Brilliant III Ultra-Fast QPCR master mix (Agilent) following manufacturer's recommendations.

Data analysis
-Quantification was based on determination of the Cq and PCR efficiency was calculated from the log-linear portion of the standard curves.
-Comparison of the efficiency of qPCR with LNA-spiked and DNA primers respectively was done by Student's T-test.

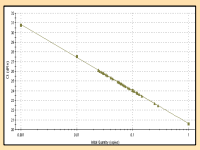
Results & Discussion

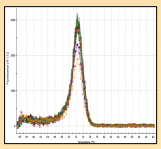
1) We tested miR-specific DNA primers by qPCR at different concentrations of synthetic miR templates. The reactions were linear over a range of eight $\log_{10}$ of synthetic template, showed one peak in melting curve analysis and good correlation between Cq and template concentration.





2) Amplification of the same miRNAs from biological samples yielded similar amplification curves as for synthetic templates and melting curve analysis showed one peak. Moreover, there was good correlation between Cq and template concentration over a four $\log_{10}$ dilutions of the biological samples ($R2 \geq 0.98$).





3) We have compared the efficiency of amplification of 18 miRNAs from porcine uterus with commercially available LNA-spiked primers sets (Exiqon) and with DNA primers respectively:

Target	Efficiency LNA primers	Efficiency DNA primers	Difference
let-7a	82%	89%	6.9%
miR-101a	85%	90%	4.9%
miR-103	93%	94%	1.6%
miR-122	95%	95%	-0.1%
miR-125b	89%	94%	4.5%
miR-139b-5p	79%	86%	6.4%
miR-150	84%	97%	12.6%
miR-195b-3p	80%	87%	7.1%
miR-20a	88%	86%	-2.0%
miR-200b	80%	94%	13.6%
miR-200c	83%	84%	0.2%
miR-21	91%	92%	1.1%
miR-23a	79%	93%	14.1%
miR-23b	81%	87%	6.2%
miR-25	84%	91%	6.7%
miR-26a	88%	96%	8.3%
miR-27a	86%	85%	-1.1%
let-7d	not specific	102%	
Average	85%	90%	5.4%

Amplification efficiencies range:

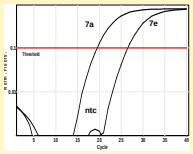
* For LNA-spiked primers: 79-95 %.
* For DNA primers: 84-102 % (significantly higher, $P<0.001$).

On average DNA primers yield 5 % higher efficiency corresponding to a 2.4 fold higher sensitivity after 30 cycles of PCR.

Possible explanations:
- In short targets such as miRNAs, the primers incorporated into the template during amplification will lead to a high proportion of LNA in the template that will decrease the efficiency of subsequent PCR cycles because nucleotide incorporation opposite to an LNA base may be difficult for some polymerases.
- the LNA-spiked primers may be more prone to form secondary structures that will lower the efficient primer concentration available to hybridize to the template.

4) We tested the ability of DNA primers to distinguish between miRNAs with a single base difference. qPCR of the specific template gave in average almost 100-fold higher signal than amplification of the template with a single base difference.

miR name	Sequence	Assay	Cq	% of specific signal
ssc-let-7a	UGAGGUAGUAGGUUGUAUAGUU	7a	19.10	100
ssc-let-7e	UGAGGUAGGAGGUUGUAUAGUU	7a	26.70	0.6 ± 0.34
ssc-let-7a	UGAGGUAGUAGGUUGUAUAGUU	7a	19.10	100
ssc-let-7f	UGAGGUAGUAGAUUGUAUAGUU	7a	26.00	1.0 ± 0.49
ssc-miR-23a	AUCACAUUGCCAGGGAUUUCCA	23a	17.30	100
ssc-miR-23b	AUCACAUUGCCAGGGAUUACCA	23a	22.30	3.1 ± 0.53
ssc-miR-125b	UCCCUGAGACCCUAACUUGUGA	125b	22.60	100
ssc-miR-125c	UCCCUGAGACCCUAACUUCGUGA	125b	29.90	0.7 ± 0.05
miR-150	UCUCCCAACCCUUGUACCAGUG	150	18.90	100
dre-miR-150	UCUCCCAACUCUUGUACCAGUG	150	26.00	0.8 ± 0.35



In fact the use of two specific primers for each miRNA allows for design of several different primer sets and therefore, it is easier to design primers with the 3' end close to the mismatch position, which is optimal for mismatch discrimination.

Conclusion

This method combines the benefits of a universal RT reaction with the specificity of two miR-specific primers for qPCR. DNA primers give significantly higher amplification efficiencies than LNA-spiked primers and the flexibility of the primer design permits to distinguish between miRNAs with a single base difference.

Hence, miR-specific quantitative RT-PCR with DNA primers is an inexpensive, highly specific, sensitive and accurate method for microRNA quantification.

References

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* Busk P.K. (2010). Method for Quantification of Small RNA Species. Patent number WO2010085966.