miR-ID®: A Sensitive and Specific Method for microRNA Quantification

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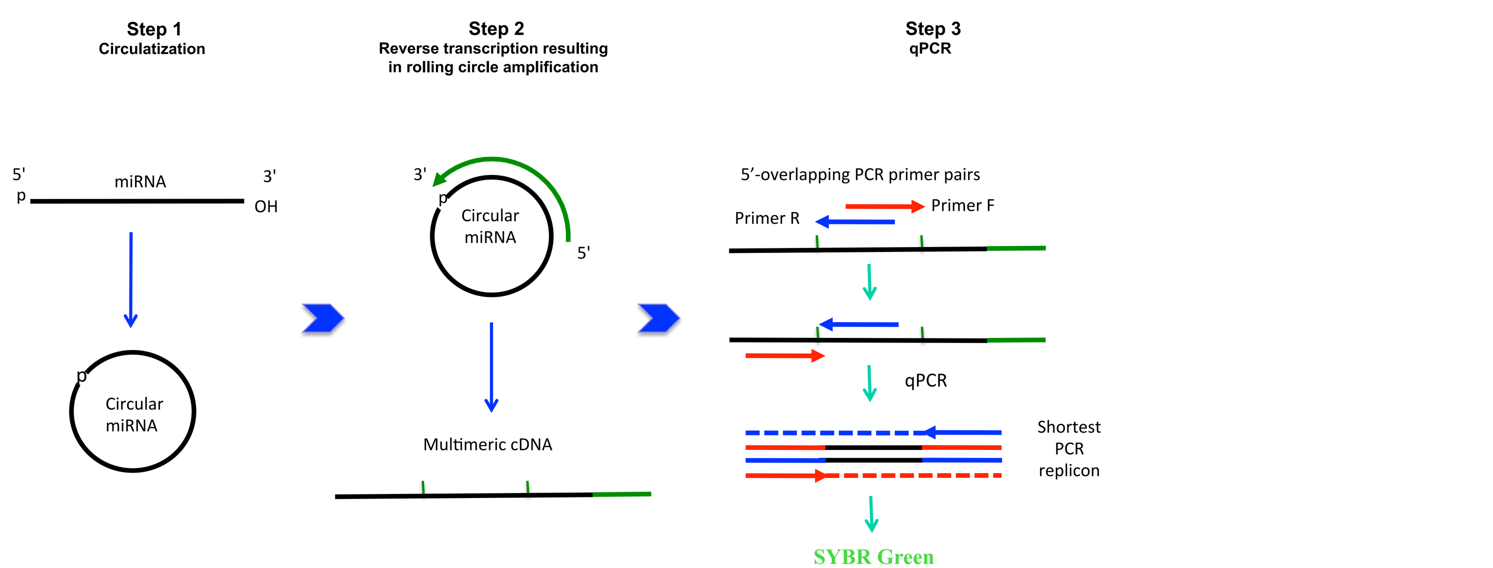
Abstract

miR-ID® is a novel platform for detecting microRNAs (miRNAs) using a circularization-based RT-qPCR method. This method is highly sensitive while using single-dye detection. miR-ID® can discriminate miRNA isoforms with single nucleotide differences at any position along the molecule as well as 2'O-methyl modifications at the 3'-terminus of miRNAs. miR-ID® works well with all sample sources, including total RNA, cell lysates, and tissue lysates.
miR-ID involves the initial circularization of linear miRNAs, followed by reverse transcription-mediated rolling circle amplification to obtain multimeric cDNA. The latter is detected by RT-qPCR with SYBR Green using highly sequence-specific 5'-overlapping primers.

Introduction

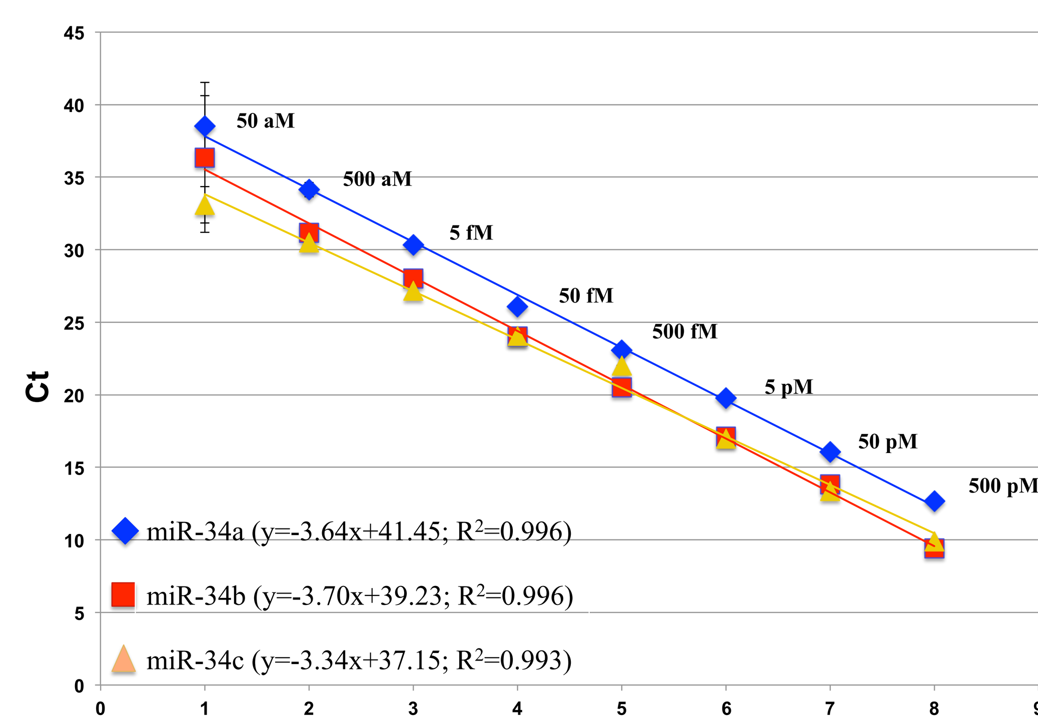
Quantification of mature miRNAs has been of interest since the significance of miRNAs in the regulation of gene expression has been recognized. Aberrant miRNA levels can be indicative of diseases and are increasingly important biomarkers for disease diagnosis and monitoring of treatment effects. qPCR is generally the method of choice to quantify low abundant, known miRNAs. TaqMan has developed reliable assays, but these are not always able to distinguish miRNAs with high sequence similarities. At similar or superior sensitivity, miR-ID addresses this gap and allows single-nucleotide discrimination at any position due to the rolling-circle generation of cDNA and highly specific overlapping PCR primers.

miR-ID® overview



•Step 1: Circularization of linear miRNAs
•Step 2: Reverse transcription mediated by rolling circle amplification to obtain multimeric cDNA
•Step 3: Quantification of the amplicon by RT-qPCR using highly sequence-specific 5'-overlapping primers and SYBR Green detection.
The alignment of the RT primers as well as that of the PCR primers can be freely moved to optimize for efficiency, sensitivity, and discrimination of closely related miRNAs.
Steps 1 and 2 of the assay can be multiplexed.

miR-ID® sensitivity

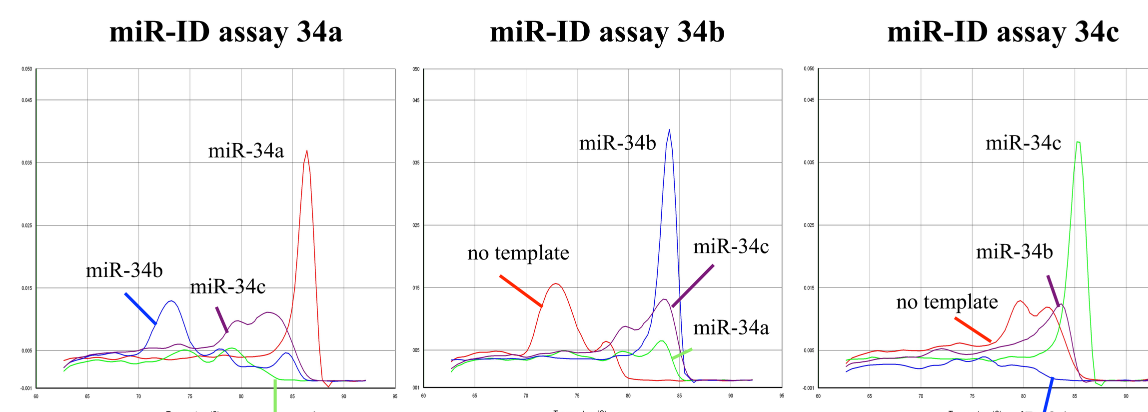


miRNA dilution curves show that miR-ID® reliably detects miRNA concentrations over an 8 log dynamic range. This range is similar to TaqMan assays. The miRNAs are miR-34a, miR-34b, and miR-34c.

miR-ID discriminates betwteen closely related miRNAs

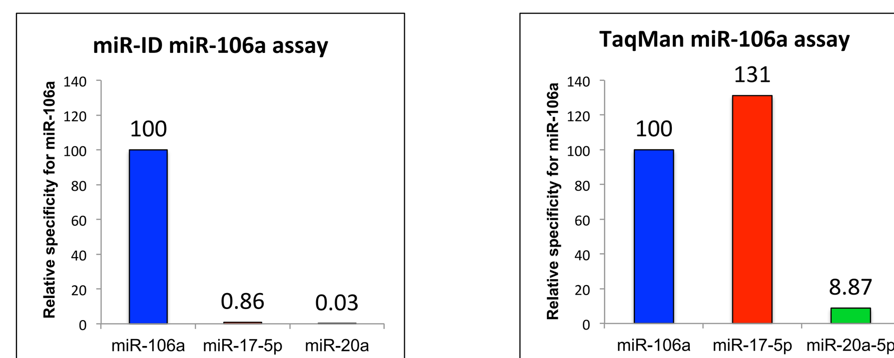
miR-34a-5p U-GGCAGUGUC-UUAGCUGGUUGU
miR-34b-5p UAGGCAGUGUCAUUAGCUGAUUG-
miR-34c-5p -AGGCAGUGUAGUUAGCUGAUUGC

	miRNA	average Ct	normalized Ct, %
miR-34a assay	34a	20.02	100.000
	34b	35.30	0.003
	34c	32.99	0.013
miR-34b assay	34a	38.32	0.000
	34b	17.04	100.000
	34c	32.83	0.002
miR-34c assay	34a	40.00	0.000
	34b	31.58	0.004
	34c	16.89	100.000



miRNAs 34a, b, and c are well discriminated by miR-ID®. For each assay, the difference in Ct value for the intended target and their isoforms was converted into a linear percentage, with the fully matched reaction normalized to 100%. All non-specific detection is well below 1% for these assays.
The dissociation curves for each assays illustrate sharp peaks expected for a single amplified product for the specific RNA and background signals for the other RNAs and no template controls.

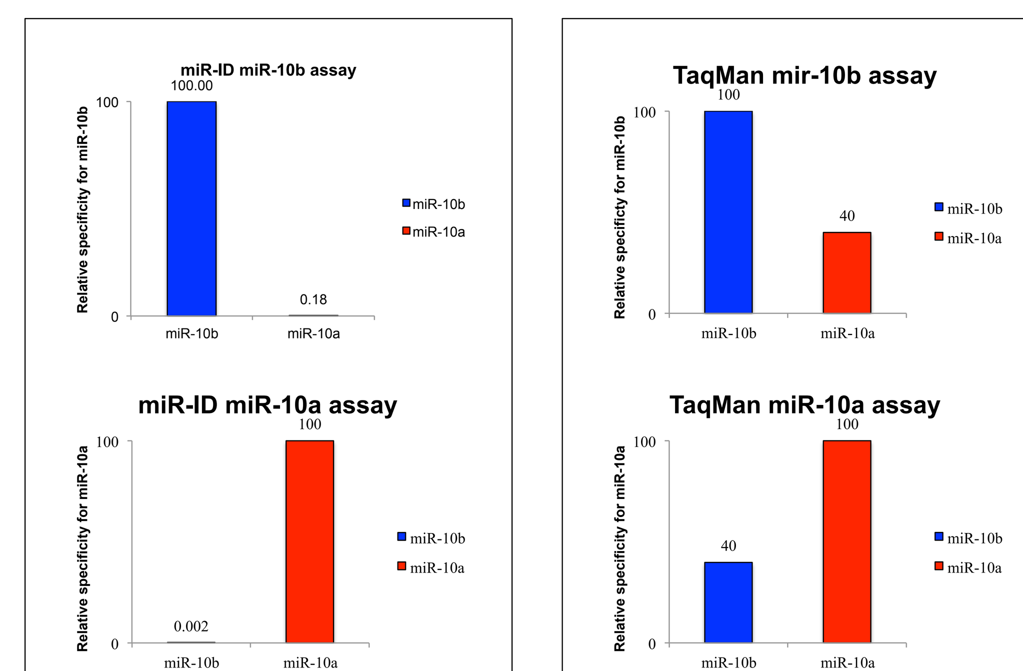
Superior discrimination of 5' end nucleotide polymorphisms by miR-ID®



106a : AAAAGUGCUUACAGUGCAGGUAG
17-5p : CAAAGUGCUUACAGUGCAGGUAG
20a-5p: UAAAGUGCUUAUAGUGCAGGUAG

Due to the circularization step and specific RT and PCR primers, miR-ID® (left panel) provides exceptional discrimination between miRNAs with single nucleotide polymorphisms at the 5' end as opposed to TaqMan assays (right panel).

Superior discrimination of nucleotide polymorphisms in the center of miRNAs by miR-ID®

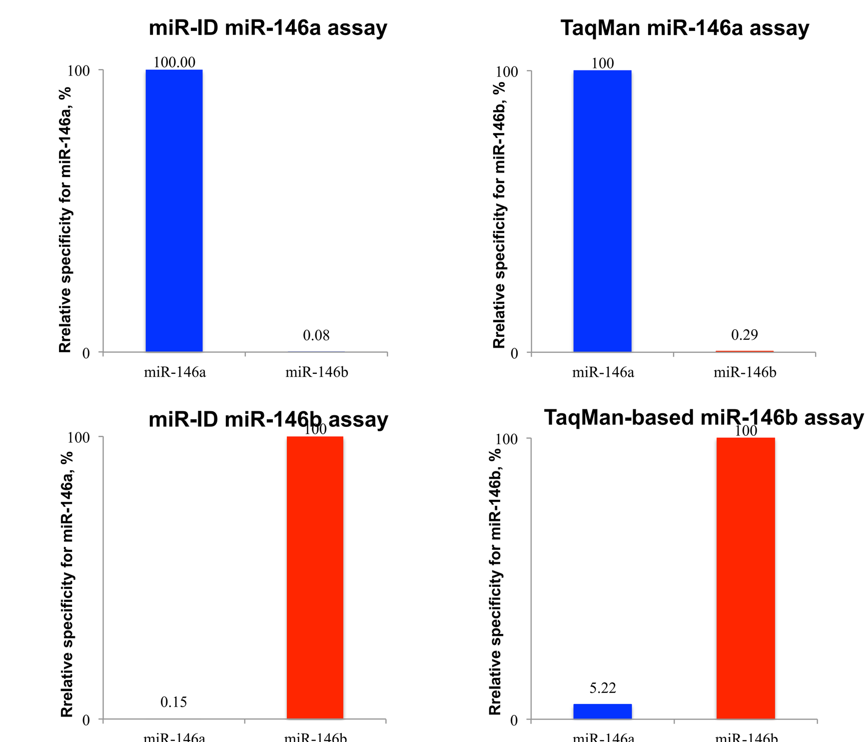


10b : UACCCUGUAGAACCGAAUUUGUG
10a : UACCCUGUAGAUCCGAAUUUGUG

miR-ID® (left panels) provides exceptional discrimination between miRNAs with single nucleotide polymorphisms at the center as opposed to the TaqMan assays (right panels).

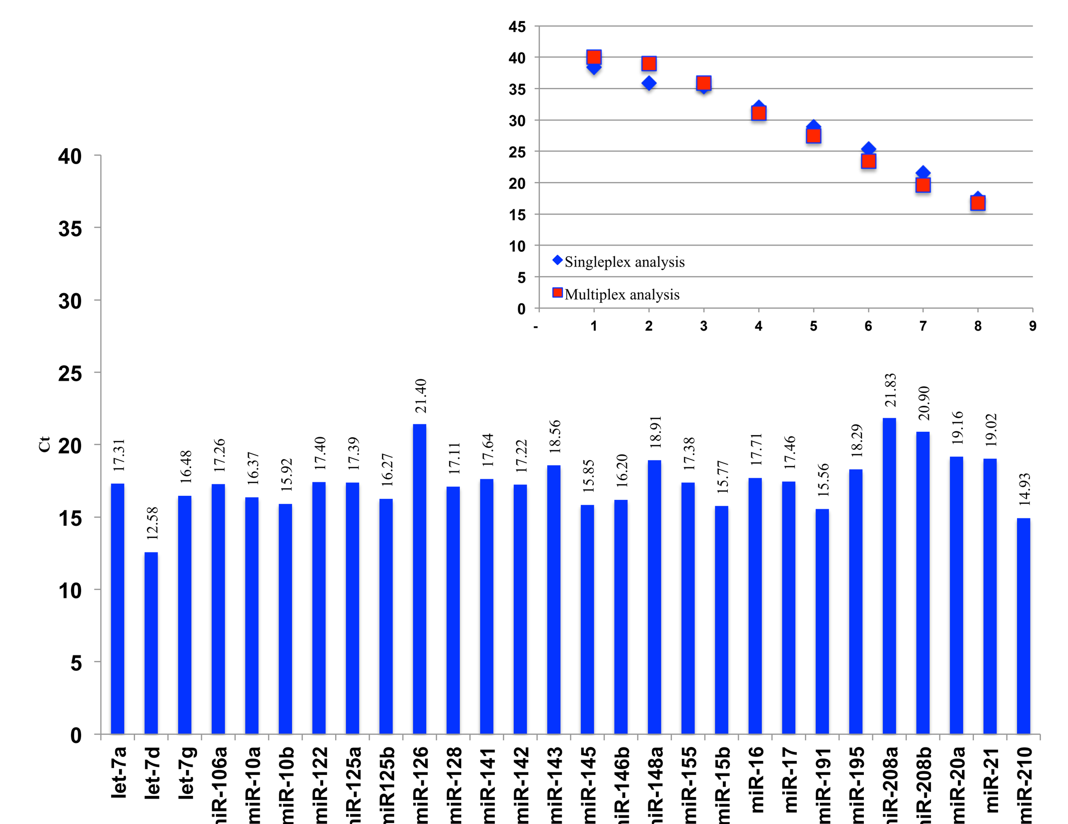
3' end nucleotide polymorphisms are well discriminated by miR-ID®

miR-146a-5p UGAGAACUGAAUUCCAUGGGUU
miR-146b-5p UGAGAACUGAAUUCCAUAGGCU



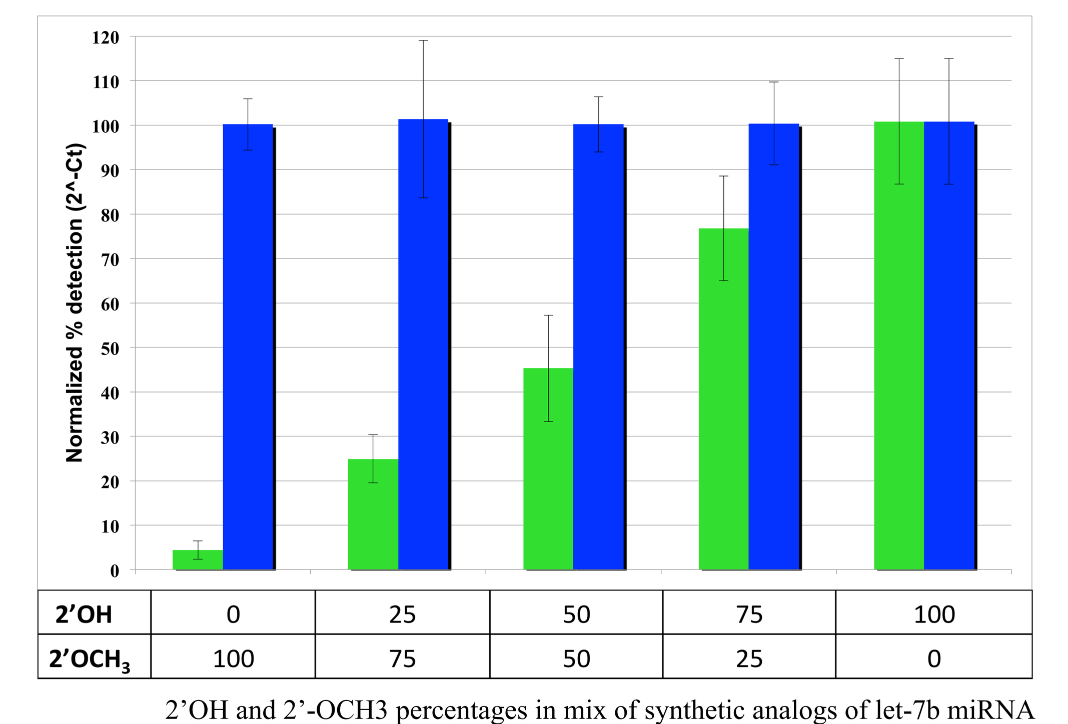
miR-ID® (left panels) discriminates well between miRNAs with single nucleotide polymorphisms at the 3' end. TaqMan assays detect up to 5.22% of the related miRNA.

Multiplex capability of miR-ID in circularization and RT steps



Thirty miRNAs from a pool of equimolar miRNAs (5 pM stock of miRXplore Universal reference, Miltenyi Biotec) were assayed in multiplex up to the qPCR step of the assay. Multiplexing circularization and reverse transcription by using a pool of specific RT primers does not impact assay quality.
Inset: comparison of miR-145a dilution curves analyzed using
a) synthetic miR-145a miRNA in singleplex (blue) or
b) using a synthetic miRNA pool in mulitplex (red)

miR-ID® distinguishes between small RNAs with 2'-OMe modifications at 3'-ends



2'OH and 2'-OCH3 percentages in mix of synthetic analogs of let-7b miRNA

The sensitivity of the circularization reaction to the 2'-OH or 2'-OMe groups at the RNA 3'-end allows to accurately and quantitatively distinguish the presence of 2'-OMe modification at the ends of small RNAs. By selecting enzymes that circularize either both types of miRNAs (2'-OMe or 2'-OH 3'termini), shown in blue, or 2'-OH termini only (green), these two end modifications can be distinguished. Here, synthetic let-7b miRNAs with the two respective 3' termini were assayed.

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Reference: Kumar, P., Johnston, B.H., Kazakov, S.A. *RNA* 17: 365-380 (2011)