Simultaneous detection of miRNA and mRNA on TaqMan Array Cards using the TaqMan Advanced miRNA workflow

This application note describes:

- Expression patterns of miRNAs and mRNAs from serum samples on Applied Biosystems™ TaqMan® Array Cards
- A protocol to detect microRNAs (miRNAs) and mRNAs from a single reverse transcription (RT) reaction using the Applied Biosystems™ TaqMan® Advanced miRNA cDNA Synthesis Kit
- High reproducibility of data on Applied Biosystems™ TaqMan® Advanced miRNA Array Cards

Introduction

miRNAs are a class of small noncoding RNAs (approximately 21 nt long) that bind complementary sequences in target mRNAs to specifically regulate gene expression. miRNAs play important roles in the regulation of gene expression during cell development, differentiation, proliferation, and apoptosis. Dysregulation of miRNAs and their targets has been associated with several diseases, including cancer. The interplay between miRNA and mRNA has been found to be important in cancer development and progression. Simultaneous expression studies of miRNA and mRNA can be valuable in understanding molecular mechanisms that may potentially have an underlying role in various diseases. In this document, we demonstrate the verification of a novel method to reverse-transcribe and preamplify miRNA and mRNA from sample-limiting serum research samples using the TaqMan Advanced miRNA cDNA Synthesis Kit.

Based on results from previous studies [1,2] and unpublished data, Dr. Buchholz's research group identified a signature of 49 mRNA and 37 miRNA targets that may help to distinguish between benign and malignant pancreatic tissue samples. In the study described here, these previously identified mRNA and miRNA targets were used to study expression pattern differences in serum from normal and test samples. Applied Biosystems™ TaqMan® Advanced miRNA Assays targeting miRNAs and TaqMan® Gene Expression Assays targeting mRNAs were spotted onto Applied Biosystems™ Custom TaqMan® Array Cards to facilitate investigation of 4 samples on each card. The results indicate that miRNAs and mRNAs can be reliably quantified from a single RT reaction.

Customer summary

Dr. Malte Buchholz is group leader and Head of Basic Research at the Center of Tumor and Immune Biology/Clinic for Gastroenterology at the University of Marburg, Germany. His research focuses on identifying novel molecular targets associated with pancreatic cancer. As part of this larger goal, one of his projects is focused on analyzing miRNAs and mRNAs in sample-limiting serum samples.

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Principle

Purified RNA from a sample containing a mixture of miRNAs and mRNAs is converted to cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Figure 1). For miRNAs, poly(A) polymerase is used to add a 3′ adenosine tail to the miRNAs; poly(A)–tailed miRNAs then undergo a 5′ adaptor ligation. This 5′ adaptor acts as the forward primer–binding site in subsequent miR-Amp reactions. Universal RT primer binds to the 3′ poly(A) tail of miRNA and converts it to cDNA. For mRNAs, the RT reaction is facilitated by a universal RT primer and random hexamers. The resulting cDNA undergoes a universal miR-Amp reaction or gene-specific preamplification reaction. The products are detected by qPCR using either gene-specific TaqMan Gene Expression Assays or miRNA-specific TaqMan Advanced miRNA Assays.

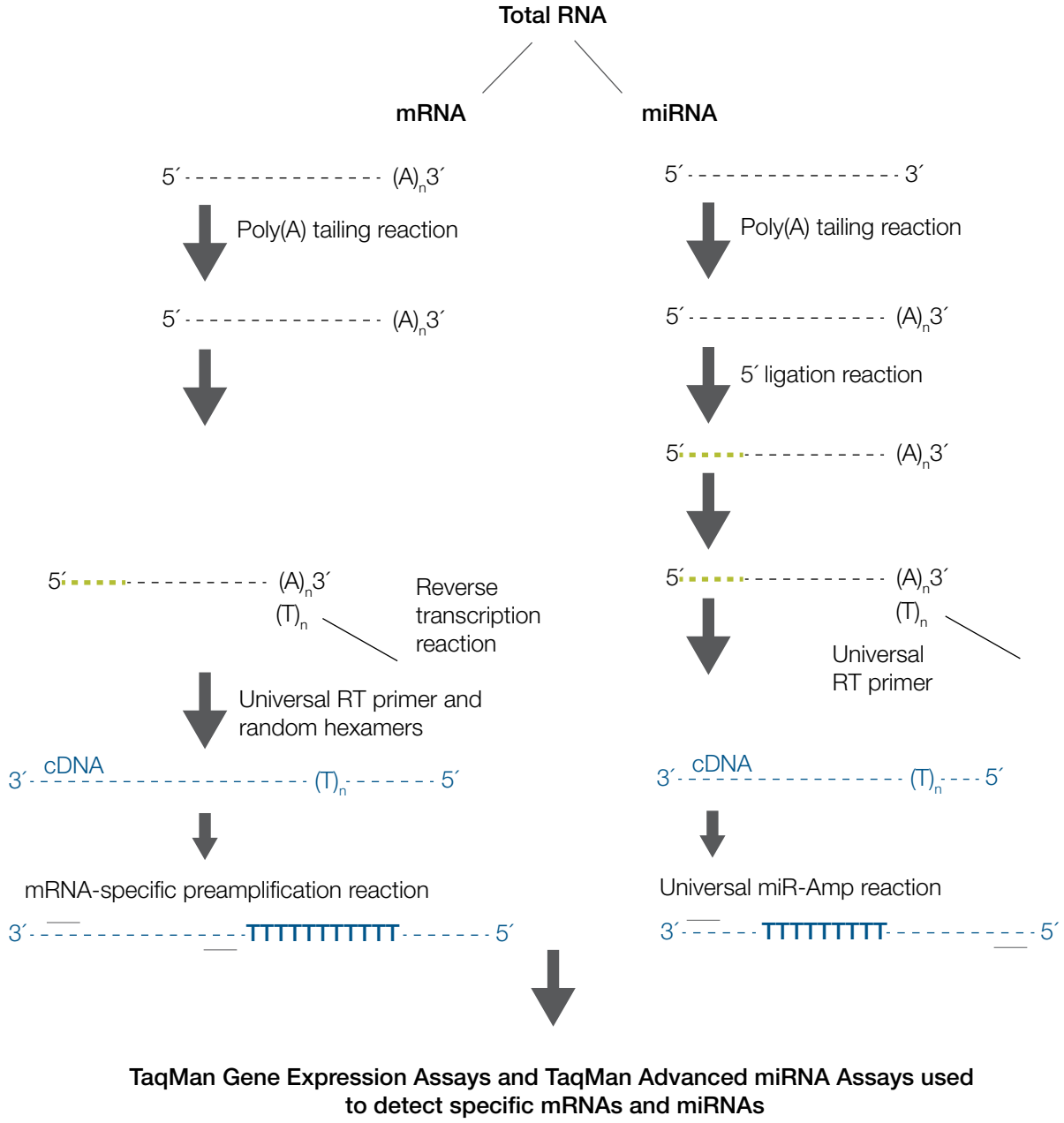

Figure 1. Flowchart showing conversion of miRNA and mRNA into cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit.

Materials and methods

Serum samples were obtained from 14 individuals with clinically manifested pancreatic ductal adenocarcinoma (PDAC; n = 14) or chronic pancreatitis, a benign inflammatory disease of the pancreas (CP; n = 2) and stored at –80°C until RNA extraction. The Invitrogen™ *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit (Cat. No. AM1556) was used to extract total RNA, including microRNA and mRNA, from 200 µL of each serum sample according to the user guide.

A total of 2 µL RNA was reverse-transcribed using the TaqMan Advanced miRNA cDNA Synthesis Kit. Poly(A) tailing and adapter ligation were performed as described in the TaqMan Advanced miRNA Assays User Guide for TaqMan Array Cards [3]. The RT and miR-Amp reactions were performed with the following protocol modifications to enable simultaneous detection of mRNA and miRNA.

RT reaction

1. In a 1.5 mL microcentrifuge tube, prepare sufficient RT reaction mix for the required number of reactions according to the following table:

Components	Volume for 1 sample (µL)	Volume for 4 samples (µL)*	Volume for 10 samples (µL)*
5X RT Buffer	6.0	26.4	66.0
dNTP Mix, 100 mM	1.2	5.3	13.2
50 µM random hexamers	1.0	4.4	11.0
20X Universal RT Primer	1.5	6.6	16.5
10X RT Enzyme Mix	3.0	13.2	33.0
Nuclease-free water	2.3	10.1	25.3
Total	15.0	66.0	165.0

* Includes 10% excess to account for volume loss from pipetting.

2. Vortex the RT reaction mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 15 µL of the RT reaction mix to each well of the reaction plate or each reaction tube containing the adaptor ligation reaction product. The total volume should be 30 µL per well or tube.

4. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

5. Centrifuge the reaction plate or tubes briefly to spin down the contents.

6. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

Step	Temperature	Time
Reverse transcription	42°C	15 min
Stop reaction	85°C	5 min
Hold	4°C	Hold

7. Proceed to the miR-Amp and preamplification reaction or store cDNA at –20ºC for up to 2 months.

miR-Amp and preamplification reaction

1. In a 1.5 mL microcentrifuge tube, prepare sufficient miR-Amp reaction mix for the required number of reactions according to the following table:

Components	Volume for 1 sample (µL)	Volume for 4 samples (µL)*	Volume for 10 samples (µL)*
2X miR-Amp Master Mix	25.0	110.0	275.0
20X miR-Amp Primer Mix	2.5	11.0	27.5
0.2X pooled TaqMan Assays	12.5	55.0	137.5
Nuclease-free water	5.0	22.0	55.0
Total	45.0	198.0	495.0

* Includes 10% excess to account for volume loss from pipetting.

2. Vortex the miR-Amp and preamplification reaction mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 45 µL of the miR-Amp and preamplification reaction mix to each well of a new reaction plate or reaction tube.

4. Add 5 µL of the RT reaction product to each reaction well or each reaction tube. The total volume should be 50 µL per well or tube.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

6. Centrifuge the reaction plate or tubes briefly to spin down the contents.

7. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings, maximum ramp speed, and standard cycling:

Step	Temperature	Time	Cycles
Enzyme activation	95°C	10 min	1
Denature	95°C	15 sec	14
Anneal and extend	60°C	4 min	
Stop reaction	99°C	10 min	1
Hold	4°C	∞	1

8. Proceed to performing the real-time PCR or store amplified cDNA at –20°C for up to 2 months. Dilute the miR-Amp reaction 10-fold before use in the qPCR reaction.

Custom TaqMan Array Cards (format 96a, Cat. No. 4342259) that combine assays for 49 mRNAs (including 18S rRNA as control) and 37 miRNAs (including 4 miRNA controls) on the same card were used for analysis. This format allows investigation of up to 4 samples in parallel. For qPCR reaction setup and loading of TaqMan Array Cards, please refer to the TaqMan Advanced miRNA Assays User Guide for TaqMan Array Cards [3].

Data analysis

qPCR data files were loaded as a project into the qPCR Relative Quantification app on Thermo Fisher Cloud. Amplification data were captured using the relative threshold method [4] with a C_{rt} cutoff at 32. Data across each Custom TaqMan Array Card were normalized using the global mean normalization method [5].

Results

TaqMan Array Cards are 384-well microfluidic cards designed to perform 384 simultaneous real-time PCR reactions using a streamlined workflow without the need for automation. TaqMan Advanced miRNA Array Cards are preloaded with dried-down TaqMan Advanced miRNA Assays. The design of TaqMan Array Cards enables users to achieve high-quality, reproducible data in a very short period of time. To demonstrate reproducibility of qPCR data in very small volumes, we loaded 2 Applied Biosystems™ TaqMan® Advanced miRNA Human A Cards with cDNA prepared from 10 ng of Invitrogen™ Human Brain Total RNA (Cat. No. AM7962). Figure 2 shows remarkable concordance of data across the entire range of C_{rt} values.

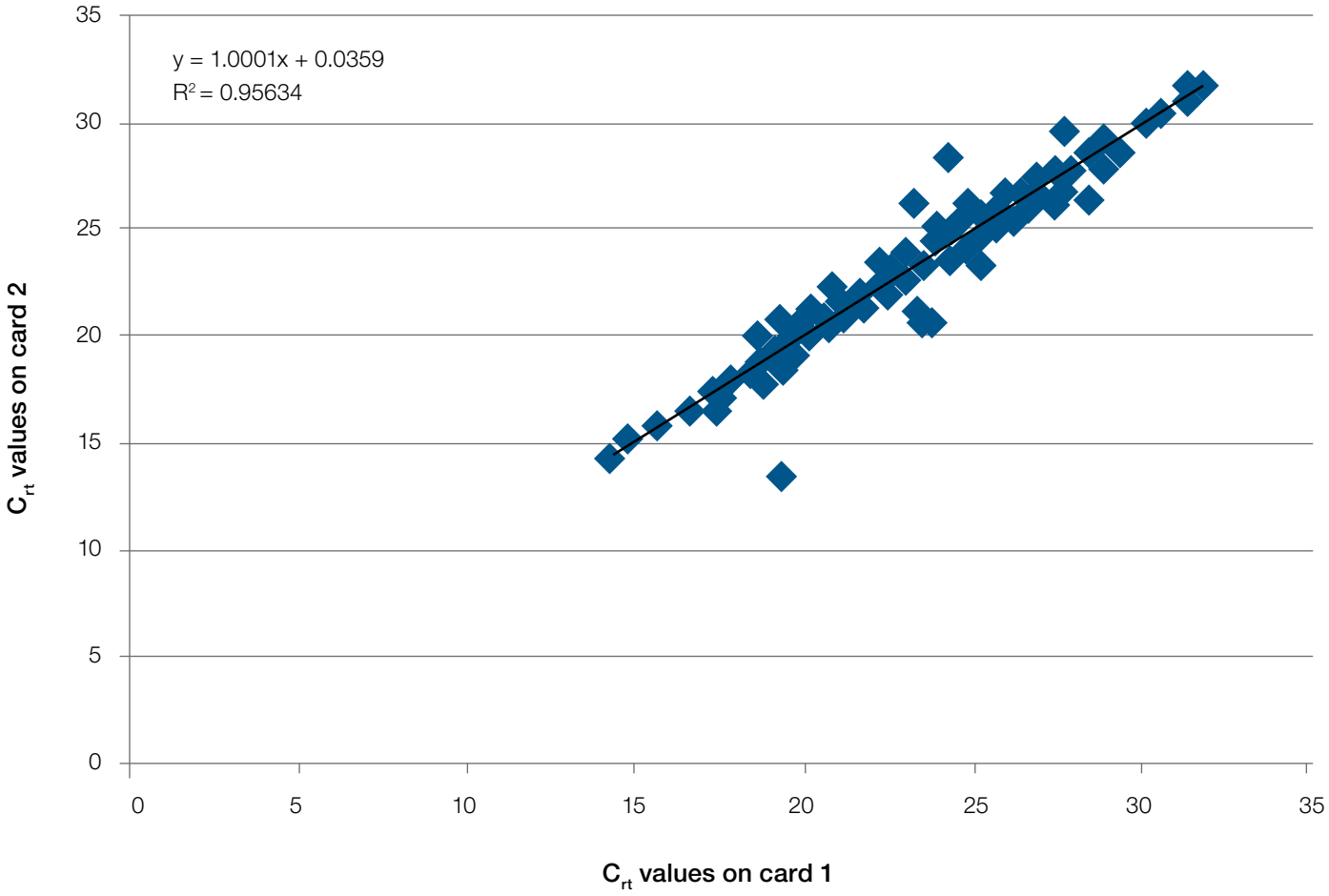

Figure 2. Reproducibility of C_t values from brain reference RNA profiled on TaqMan Advanced miRNA Array Cards.

The methods discussed in this document enable detection of both miRNAs and mRNAs on the same TaqMan Array Card. Custom TaqMan Array Cards were spotted with 49 TaqMan Gene Expression Assays and 37 TaqMan Advanced miRNA Assays, which were previously identified as significant in pancreatic tissues. Figure 3 shows the expression pattern of these miRNA and mRNA targets in 16 different serum samples. A total of 14 of the 16 samples represent PDAC while 2 represent serum samples from chronic pancreatitis. Pancreatic tumors are known to display a large degree of intertumoral heterogeneity [6], which is reflected by the diverse expression patterns of the selected marker set. Clinical and radiographic tests of tissues make differentiation of CP and PDAC not only difficult but sometimes also impossible. Nonetheless, chronic pancreatitis samples (CP1 and CP2) can readily be separated from the spectrum of patterns observed in PDAC as evidenced by the dendrogram.

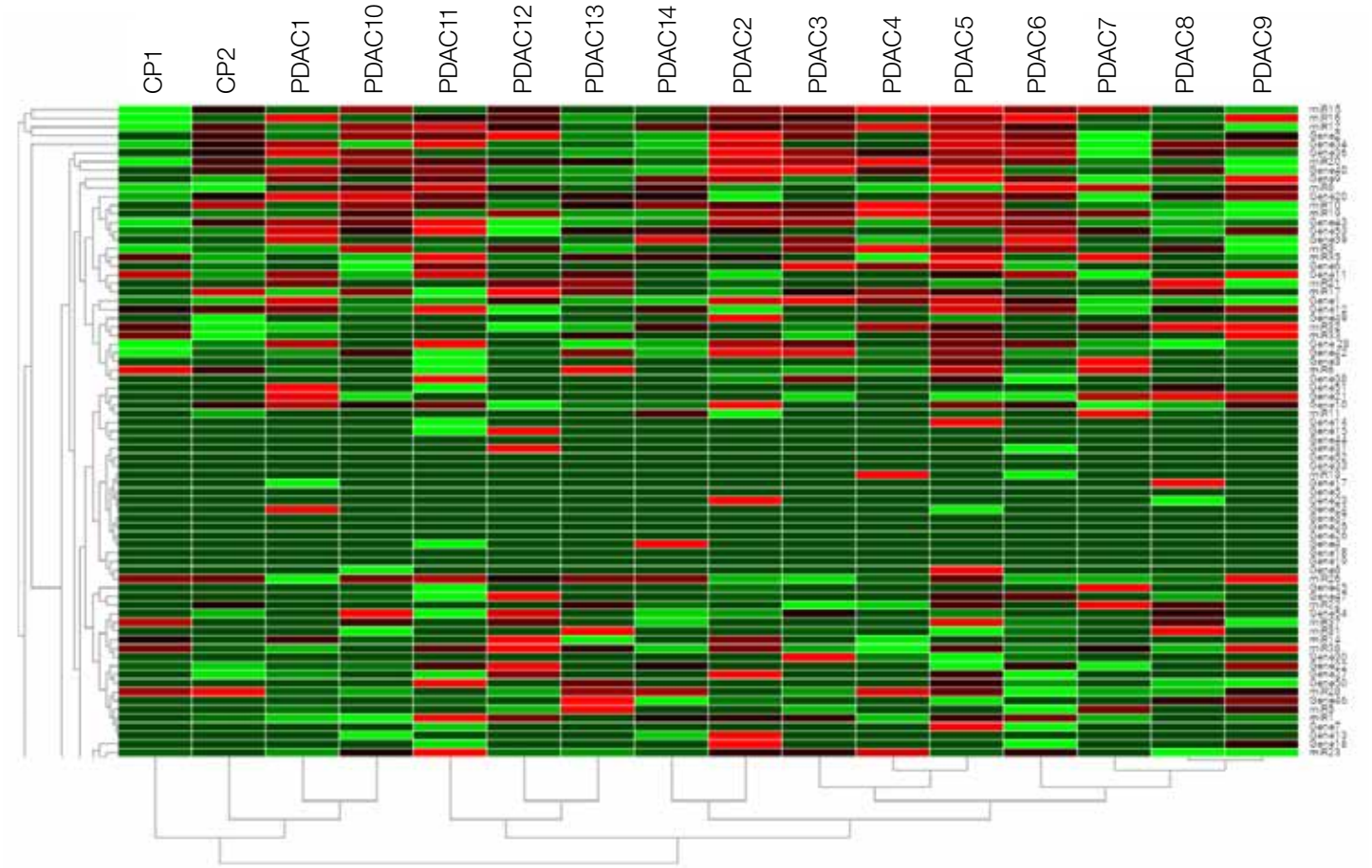

Figure 3. Euclidean distance average linkage cluster analysis generated with ΔC_{rt} values obtained from custom arrays containing TaqMan Assays for mRNA and miRNA.

Conclusions

PDAC is among the tumors with the highest degree of intertumoral genetic heterogeneity, suggesting that no single molecular biomarker and no single molecular targeted therapy is sufficient for identification of PDAC. Accurate molecular differentiation of pancreatic cancers will potentially require a high degree of multiplexing of markers to account for these variabilities. In addition, future tests should place as little burden on the patient as possible. Analysis of liquid biopsies (body fluids such as serum, urine, and duodenal fluid) is highly preferred, but this comes at the cost of low yield of test samples.

In previous work, Dr. Buchholz's group identified sets of serum mRNA and miRNA markers that each provide valuable and nonredundant information to help make the important distinction between PDAC and other, much less severe diseases. The mixed, custom TaqMan Array Cards and the protocols described in this document offer an ideal system for Dr. Buchholz's team to simultaneously analyze both types of markers with high sensitivity and precision. TaqMan Array Cards also provide a highly standardized workflow with limited hands-on time for qPCR in small 1 µL reaction volumes. These combined benefits can help facilitate research on the relationship between miRNA and mRNA during cancer progression.

The ability to analyze miRNAs and mRNAs simultaneously opens exciting avenues into the interplay of regulatory miRNAs and gene expression. However, since analysis of the permutations and combinations of the possible miRNA–mRNA reagent interactions is not always feasible, we recommend that the adoption of the protocol described above be followed by a verification study.

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Ordering information

Product	Cat. No.
TaqMan Advanced miRNA cDNA Synthesis Kit	A28007
TaqMan Fast Advanced Master Mix	4444557
TaqMan Advanced miRNA Human A and B Card	A31805
TaqMan Advanced miRNA Human A Card	A34714
TaqMan Advanced miRNA Human B Card	A34715
TaqMan Advanced miRNA Human Control Card	A34716
TaqMan Advanced miRNA Human Serum Plasma	A34717

References

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2. Gress TM, Kestler HA, Lausser L et al. (2012) Differentiation of multiple types of pancreatico-biliary tumors by molecular analysis of clinical specimens. *J Mol Med* (Berl) 90(4):457–464.
3. TaqMan Advanced miRNA Assays User Guide for TaqMan Array Cards, Pub. No. MAN0016122.
4. C_{rt}, a relative threshold method for qPCR data analysis on the QuantStudio 12K Flex system with OpenArray technology. Application note available at thermofisher.com, Pub. No. CO020269.
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