Expression analysis of both mRNA and miRNA on the same TaqMan® Array Card

Introduction

Expression profiles of both mRNAs and miRNAs have become important markers for the characterization of specific cell types, disease progress, or tumor types. Often, both types of molecules are needed for exact classification of a certain state; thus, a method for the simultaneous detection of both mRNA and miRNA in one step would be a very valuable tool in molecular detection technologies. This application note describes a protocol developed with Professor Thomas Gress from the Clinic for Gastroenterology, University Clinic Marburg, Germany, for the measurement of mRNA and miRNA in the same sample. In this method, the researchers combined the samples together in one reverse transcription (RT) reaction. The cDNA was preamplified and the expression of specific mRNA and miRNA were analyzed using a single Custom TaqMan® Array Card. They showed that they can simultaneously reverse-transcribe and reliably preamplify and detect both RNA species at once; the method is simple and provides a robust protocol to quickly get results.
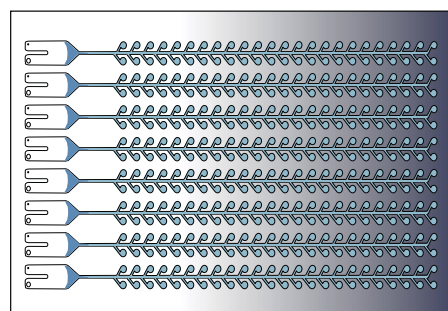

Figure 1. **Experimental setup for the TaqMan® Array Cards.** Total RNA was extracted from 5 pancreatic cancer cell lines and pooled. Reverse transcription was performed without (sample A) and with (sample B) a subsequent preamplification step. Expression of the mRNAs and miRNAs was then analyzed using TaqMan® Array Cards containing 79 TaqMan® Gene Expression Assays plus 6 endogenous control assays; and 9 TaqMan® miRNA Assays plus 2 miRNA endogenous control assays. The same experiment was repeated on a second TaqMan® Array Card on Day 2, starting again from the original total RNA sample. Including technical replicates, this gave 4 x 96 data points for both sample A and sample B.

The TaqMan® Array Card, designed to be used with the QuantStudio®, ViiA™ 7 , and 7900HT Fast Real-Time PCR systems, is a real-time PCR consumable consisting of 384 wells connected by a series of microfluidic channels. Selected TaqMan® Assays (gene expression or miRNA assays) are loaded and dried in the array wells as specified by the user. Up to 8 cDNA samples per array are added to 8 loading ports, allowing delivery of a single sample-specific reaction mix to 48 wells (or, in this case, 4 samples to 96 wells (Figure 1)). TaqMan® Array Cards provide a standardized format for gene expression studies that permits direct comparison of results among different researchers and laboratories.

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Professor Gress is coordinating a nationwide Translational Genome Research Network in Pancreatic Cancer. A subgroup of this network, led by principal investigator Dr. Malte Buchholz, is aimed at the development of a molecular tool for the characterization of pancreatic tumors.

In a previous study [1], Buchholz et al. developed a specialized cDNA array and demonstrated that expression of a 169-gene signature was sufficient to distinguish malignant from benign pancreatic tissue with high accuracy. Others have reported similar findings. More recently, Buchholz and his research team have configured a Custom TaqMan® Array Card (format 96a, Cat. No. 4342259) that combines 79 mRNAs (plus 5 mRNA controls and 18S rRNA) and 9 miRNAs (plus 2 small RNA controls) on the same card. This format allows them to run up to 4 samples in parallel. The team has also successfully validated a method to reverse-transcribe both mRNAs and miRNAs in a single reaction tube, followed by preamplification of the reverse transcription products. In this publication, they have first demonstrated the technical validation of their method using a pool of RNA from five different pancreatic cancer cell lines, and then evaluated the performance, reproducibility, and accuracy of these "mixed" TaqMan® Array Cards for the analysis of precious samples.

Materials and methods

Samples: Total RNA from five pancreatic cancer cell lines was isolated using an RNeasy® kit (Qiagen), then pooled together. Total RNA samples were also obtained from different malignant and benign pancreatic tumor samples.

Reverse transcription, preamplification, and qPCR:

50 ng of total RNA from each sample was reverse-transcribed using the TaqMan® microRNA Reverse Transcription Kit (Cat. No. 4366597) and a custom microRNA RT primer pool and mRNA/miRNA PreAmp primer pool. The RT products were preamplified using the custom mRNA/miRNA PreAmp primer pool, and the preamplification products were analyzed on a TaqMan® Array Card run on a 7900 Fast real-time PCR instrument. See the Appendix for details.

Validation of PreAmp reaction

Preamplification of cDNA prior to real-time PCR is performed through limited cyclic amplification with gene-specific preamplification primer pools for both mRNAs and miRNAs combined together. If performed carefully, this step serves to preamplify small amounts of cDNA without introducing amplification bias to the sample. By employing a preamplification step, researchers can successfully perform gene expression analyses using samples that are available only in limited amounts, including formalin-fixed, paraffin-embedded (FFPE) tissues, laser-capture microdissection (LCM), and other precious samples.

To investigate if or to what extent an amplification bias is introduced during the preamplification step, the following experiments were performed. First, total RNA pooled together from 5 cancer cell lines was reverse-transcribed (sample A). Then, starting with the same total RNA pool, another sample (sample B) was reverse-transcribed and then preamplified. Both samples A and B were run in technical replicates on the same TaqMan® Array Card (Figure 1). This experiment was repeated on another day, starting with the same RNA pool. This produced 4 x 96 data points for sample A and for sample B. Figure 1 shows a diagram of the experimental setup. In addition, 4 RNA samples obtained from different malignant and benign pancreatic tumor samples were reverse-transcribed and preamplified, and expression of miRNAs and mRNAs was analyzed using the TaqMan® Array Card. These samples were run in single reactions on 1 card (see Figure 2 for tissue types).

Expression profile analysis

The expression profiles of the 79 mRNAs/9 miRNAs (plus 8 controls) from samples A and B and from four pancreatic cancer tissue samples are displayed in Figure 2. C_t values were calculated by SDS Software v2.3. Relative expression levels for each mRNA and miRNA were calculated ($2^{-\Delta C_t}$ method), using the mean C_t values of the mRNA or miRNA controls as respective internal references. Relative expression values were mean-centered, and heat maps were generated using the Cluster and TreeView software tools [2].
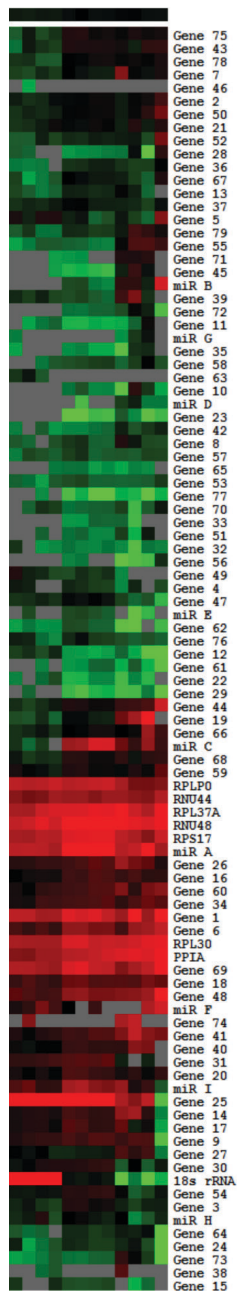
Figure 2. Hierarchical clustering analysis of expression data for RNA samples across 79 mRNAs and 9 miRNAs. Relative expression values ($2^{-\Delta C_t}$) of replicates of sample A (1–4, no preamplification), sample B (5–8, preamplification), and 4 pancreatic tissue samples (9–12: intraductal papillary mucinous neoplasm, pancreatic ductal adenocarcinoma, chronic pancreatitis, solid pseudopapillary tumor) were mean-centered and heat maps were generated. Green: low expression; red: high expression; gray: not detected.

The expression data obtained from samples A and B processed on 2 different days with 2 technical replicates were compared to each other and to the expression data obtained from the 4 pancreatic tumor samples. The results can be summarized as follows:

- The closest clustering was observed between the technical replicates (Figure 2: compare replicate 1 vs. 2, 3 vs. 4, etc.)

- Identical samples run on different days cluster very closely as well (true for both samples A and B; see Figure 2: replicates 1/2 vs. 3/4)

- Expression data from the RNA pools derived from cell culture samples A and B (both without and with preamplification) cluster closer to each other (i.e., are more similar to each other) than to those arising from the tissue samples

- The 4 tissue samples fall into 2 groups: three of the tissue samples are quite similar to each other, while the fourth (solid pseudopapillary tumor) shows a much lower degree of similarity, reflecting the associated characteristics of these tumor types

Overall, the results were as expected. Data from the technical replicates and similar samples cluster very closely, and the number of expressed genes is higher in the preamplified samples (5–8) compared to the nonpreamplified samples (1–4). Unfortunately (but not unexpectedly), some of the miRNA candidates are not detected in the pooled RNA from cultured cell lines (e.g., miRNA G and miRNA D, Figure 2). The tissues were chosen as positive controls, especially for the detection of the miRNAs.

Statistical analysis

To analyze the reproducibility of the replicates in more detail and to investigate whether a bias is introduced by using the PreAmp method, the negtrend statistical method [3] was applied, using the exported C_t values as input data.

Reproducibility—same sample on different days

The reproducibility of identical RNA samples (i.e., comparison of sample A with sample A and sample B with sample B), run on different days (mRNA only) was assessed. For each analyzed gene, five ΔC_t (C_t mRNA – C_t endogenous control) values were generated using the C_t values of the five different endogenous controls that were detected on the TaqMan® Array Cards. That gave 10 ΔC_t values for each gene analyzed in sample A on day 1 (2 technical replicates) and another 10 ΔC_t values for each gene analyzed in sample A on day 2. The null hypothesis assumes that the results of the two groups are identical and that there is no significant difference between the groups. The negtrend algorithm compares the 10 ΔC_t values for each gene in these two groups. If there is no significant difference between the two groups, the null hypothesis is true.

Table 1. Reproducibility of the same sample run on different days. The algorithm determined if there was a significant difference between samples run on day 1 or day 2.

	Algorithm calls			Percent true*
	True	False	NA	
Sample A (no PreAmp)	39	22	18	64%
Sample B (PreAmp)	53	21	5	72%

True = no difference. False = statistical difference. No C_t cutoff was applied. NA = not enough data points to apply the algorithm.
*The percentage of genes that showed no significant difference (i.e., good reproducibility) between the two samples run on different days.

The results from sample B (which included a preamplification step) show a much higher number of detected genes and a much lower NA (not enough data) rate (Table 1) than those of sample A (no preamplification step). In sample A, 64% (39 out of 61) of all detectable genes gave a "true" call, which means that no significant difference between the results on day 1 and day 2 was detected by the algorithm. With sample B the number of detectable genes was much higher than with sample A, and the percentage of genes that gave a highly reproducible result was higher as well (72%, or 53 out of 74). This demonstrates that including a preamplification step leads to more reproducibility and therefore much more reliable data analysis (Table 1).

Reproducibility—with and without preamplification step

The negtrend algorithm was applied to assess all the combined results from both sample A and sample B (including all technical replicates as well as runs on two different days). Again, 10 ΔC_t values were calculated for each gene as described above. The results were 57 true calls, 18 false calls, and 4 NA; thus, for 76% of all detected genes analyzed in this experiment, no bias was introduced by the preamplification step.

Note that since many of the miRNAs were not detectable in the RNA pools used to validate this method, only mRNA data (and not miRNA data) were included in this analysis.

Results of preamplification validation

The experiments to validate the preamplification step produced favorable results. The workflow gave reproducible results when the same sample was assayed on two different days; and when the preamplification step was included, a higher number of the genes interrogated gave a true call according to the algorithm used in the analysis. In addition, for a majority of the genes tested, no bias was introduced when a preamplification step was used. For many of the genes that gave a false call according to the algorithm, the C_t values in the no-preamplification condition were very high ($C_t > 32$) or one of the replicates failed to give a signal, which indicates that those false calls were not a direct result of a failure of the methodology per se. Taken together, these data indicate that the preamplification workflow itself is valid and that incorporation of a preamplification step offers significant improvement in the numbers of mRNAs and miRNAs detected.

Analysis of FNABs from malignant pancreatic tissues

After successful technical validation of the preamplification workflow described above, a preliminary set of 17 FNAB samples (10 from malignant tumors, 7 from benign chronic pancreatitis cases) was analyzed using the "mixed" TaqMan® Array Cards method. The samples were obtained from freshly resected surgical specimens under standard informed consent procedures. This allowed the researchers to ensure realistic simulation of routine FNAB procedures and avoid sampling error, and facilitated accurate reference analysis by histopathological evaluation of the resected specimens.
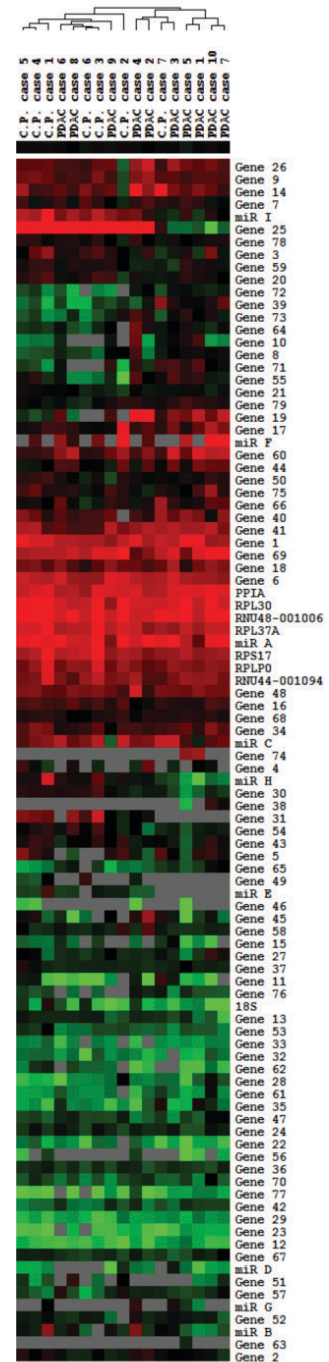

Figure 3. Hierarchical cluster analysis of 17 FNABs. CP: chronic pancreatitis; PDAC: pancreatic ductal adenocarcinoma.

The collected tissues were stored until RNA isolation could be carried out, and RNA was isolated from each one using a commercially available nucleic extraction kit according to the manufacturer's instructions. Total RNA samples were stored until cDNA synthesis was performed. cDNA synthesis and preamplification were performed according to the protocol described in the Appendix.

Each cDNA was analyzed by real-time PCR on the mixed TaqMan® Array Cards, and C_t values for target genes were obtained. All C_t values were normalized to the mean C_t values of the mRNA or miRNA controls, and ΔC_t values were calculated. Relative expression values ($2^{-\Delta C_t}$) were plotted and compared. Unsupervised two-dimensional hierarchical cluster analysis of relative gene expression data ($2^{-\Delta C_t}$ method) reveals distinct clusters of pancreatic cancer (PDAC) samples and benign control (CP) samples (Figure 3).

Conclusion
This application note demonstrates the success of a research method developed for the simultaneous detection of pancreatic cancer–related mRNAs and miRNAs on TaqMan® Array Cards. Included in the method are the steps to perform simultaneous reverse transcription and preamplification of the targets (up to 85 mRNAs and 11 miRNAs) and a validation procedure to show that bias was not introduced during preamplification for the majority of analyzed targets. The mixed TaqMan® Array Card method has proven to give reliable results and is very easy to use. Although further work is needed for their study, Dr. Buchholz and his team agreed that the preliminary results were very promising. The performance of the TaqMan® Array Card with both miRNA and mRNA TaqMan® Assays was excellent, providing high levels of reproducibility, sensitivity, and specificity. The workflow combining mRNA and miRNA gene analysis and running on a single TaqMan® Array Card "represents tremendous progress in our experimental approach," Dr. Buchholz concluded.

Acknowledgment

References
1. Buchholz M, Kestler HA, Bauer A et al. (2005) Specialized DNA arrays for the differentiation of pancreatic tumors. *Clin Cancer Res* 11:8048–8054.

2. Eisen MB, Spellman PT, Brown PO et al. (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 95:14863–14868.

3. Dixon PM, Pechmann JHK. (2005) A statistical test to show negligible trend. *Ecology* 86:1751–1756.

Appendix—experimental methods
Step 1: cDNA synthesis
Materials needed
- Custom microRNA RT primer pool
- Custom mRNA/miRNA PreAmp primer pool
- Total RNA (1–250 ng)
- TaqMan® microRNA Reverse Transcription Kit (Cat. No. 4366597)
- $MgCl_2$ (100 mL, Cat. No. AM9530G)

Preparing the RT reactions
Prepare the RT reaction mix in a tube. All reagents and reactions, including the RT reaction mix, should be prepared on ice. Do NOT vortex. The final volume of each RT reaction should be 10 µL.
1. Dispense 3.5 µL of RT reaction mix per well or tube (prepared according to the table above).
2. Add 2 µL of the custom microRNA RT primer pool.
3. Add 2 µL of the custom mRNA/miRNA PreAmp primer pool.

RT reaction mix

Component	Stock	Final	Volume for one reaction	Volume needed in 20-reaction mix*
dNTPs with dTTP	25 mM each	0.5 mM	0.20 µL	4.8 µL
MultiScribe™ Reverse Transcriptase	50 U/µL	10 U/µL	2.00 µL	48.0 µL
10X RT Buffer	10X	1X	1.00 µL	24.0 µL
1 M $MgCl_2$	1 M	3 mM	0.03 µL	0.7 µL
AB RNase Inhibitor	20 U/µL	0.25 U/µL	0.13 µL	3.0 µL
H_2O			0.15 µL	3.5 µL
Total volume			3.50 µL	84.0 µL

*Volumes listed include ~20% excess.

4. Add 2.5 µL of total RNA (RNA concentration is 20 ng/µL).

5. Seal tube or plate, mix by inverting the plate or tube, and then spin briefly.

6. Incubate on ice for 5 min.

RT thermal cycling protocol

	16°C, 2 min
Cycle (40 cycles)	42°C, 1 min
	50°C, 1 sec
Denature	85°C, 5 min
Hold	4°C, indefinitely

The RT product is stable at –20°C for at least a week.

Step 2: Preamplification

Materials needed
• TaqMan® PreAmp Master Mix (Cat. No. 4384266)

• Custom mRNA/miRNA PreAmp primer pool

Preparing the preamplification reactions
Prepare the PreAmp primer and enzyme mix in a tube. All reagents and reactions should be prepared on ice. Do NOT vortex. The final volume of each PreAmp reaction should be 25 µL.

PreAmp primer and enzyme mix

Component	Stock	Final	Volume for one reaction	Volume needed for 20 25-µL reactions*
TaqMan® PreAmp Master Mix	2X	1X	12.5 µL	300 µL
Custom mRNA/ miRNA PreAmp primer pool			6.25 µL	150 µL
H₂O			1.25 µL	30 µL

*Volumes listed include ~12.5% excess.

1. Aliquot 20 µL of the PreAmp primer and enzyme mix per well or tube.

2. Add 5 µL of the RT product (from step 1) per well or tube.

3. Seal the tube or plate, mix thoroughly by inverting 6 times, and then spin briefly.

PreAmp thermal cycling protocol

Denature	95°C, 10 min
Anneal	55°C, 2 min
Extend	72°C, 2 min
Cycle (12 cycles)	95°C, 15 sec
	60°C, 4 min
Hold*	99.9°C, 10 min
Hold	4°C, indefinitely

*Required for enzyme inactivation.

Use the preamplified products immediately or store at –20°C for no more than a week.

Step 3: Real-time PCR on TaqMan® Array Cards

Real-time PCR mix

Component	Volume needed for 1 sample on format 96a* TaqMan® Array Card
PreAmp product	2.25 µL
TaqMan® Universal PCR Master Mix, No AmpErase UNG (Cat. No. 4324018), 2X stock	112.50 µL
H₂O	110.25 µL
Total volume	225.00 µL*

*Volumes listed include ~20% excess.

1. Mix thoroughly and spin briefly.

2. Aliquot 100 µL per port (2 ports per sample) in a TaqMan® Array Card, seal, and spin.
   Note: Proper loading of the TaqMan® Array card is critical for successful use. See the TaqMan® Array Microfluidic Cards User Bulletin online (search for Cat. No. 4400263 at lifetechnologies.com).

3. Perform real-time PCR on the Applied Biosystems® ViiA™ 7 Real-Time PCR System (with TaqMan® Array Block) or 7900HT Fast Real-Time PCR System with TaqMan® Array Block using universal cycling conditions (95°C/10 min, then 95°C/15 sec, 60°C/60 sec for 40 cycles).

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