Methods 59 (2013) 174–178

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Review Article

Gene expression analysis of both mRNA and miRNA on the same TaqMan® Array Card
Development of a pancreatic tumor tissue classification methodology

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1. Introduction

Professor Thomas Gress from the Institute for Gastroenterology, University Clinic Marburg, Germany, is coordinating a nation-wide Translational Genome Research Network in Pancreatic Cancer. A sub-group 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 from a 169-gene signature was sufficient to distinguish malignant from benign pancreatic tissue with high accuracy. Others have reported similar findings [2]. More recently, Buchholz and his research team have configured a TaqMan® Array Card (Life Technologies), Carlsbad, CA that combines 79 mRNA genes (with 5 mRNA controls and 18S rRNA) and 9 miRNA genes (with 2 miRNA controls) on the same card. This format allows them to run up to four samples in parallel. The team have also successfully validated a method to reverse transcribe both mRNAs and miRNAs in one tube and to simultaneously preamplify these genes in one tube, as well. In this publication, they first demonstrated the technical validation of their method using a pool of cells 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 fine needle aspiration biopsy (FNAB) tissue samples.

2. Validation of preamp reaction

Preamplification of cDNA prior to real-time PCR is performed through limited cyclic amplification with gene-specific preamp primer pools for both mRNAs and miRNAs. 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 needle biopsies.

To investigate if or to which extent an amplification bias is introduced during the preamplification step, the following experiments were performed: total RNA was isolated from five pancreatic cancer cell lines, pooled together, and reverse transcribed (see Appendix A for method). Expression of mRNAs and miRNAs was analyzed using a TaqMan® Array Card (sample A). Starting from the same RNA pool, a sample B was reverse transcribed and preamplified (see Appendix A for method) and analyzed on the same TaqMan Array Card. Each sample was run in technical duplicates. This experiment was repeated on another day, starting from the same RNA pool. This produced 4 × 96 data points for sample A and for sample B. Fig. 1 shows a diagram of the experimental setup. In addition, 4 RNA samples obtained from different malignant and benign pancreatic tumor biopsies were reverse transcribed and expression of miRNA and mRNA was analyzed using the TaqMan® Array Card. These samples were run in single reactions (one card with 4 samples, see Fig. 2 for description of tissue types).

3. Gene expression profile analysis

The gene expression profiles of the 79 mRNAs/9 miRNAs (plus 8 controls) as detected using TaqMan® Array Card analysis of sample A, sample B, and from four pancreatic cancer tissue samples are displayed in Fig. 2. C_t values were calculated by SDS Software v2.3 and exported. Relative expression levels for each mRNA and miRNA gene were calculated ($2^{-\Delta Ct}$ method), using the mean C_t value of the mRNA or miRNA controls as internal reference, respectively. Relative expression values were mean-centered and heat maps generated using the "Cluster" and "TreeView" software tools [3].

The gene expression data obtained from samples A and B processed on two different days in two technical replicates were compared to the gene expression data obtained from the four biopsy samples obtained from pancreatic tumors (run on a separate TaqMan® Array Card), in order to compare the *in vitro* cell line data with results from the relevant samples. The results can be summarized as follows:

- The closest gene expression data clustering was observed between the technical replicates (Fig. 2, compare samples 1 + 2 and 3 + 4, etc.).

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1046-2023/$ - see front matter
http://dx.doi.org/10.1016/j.ymeth.2012.09.014

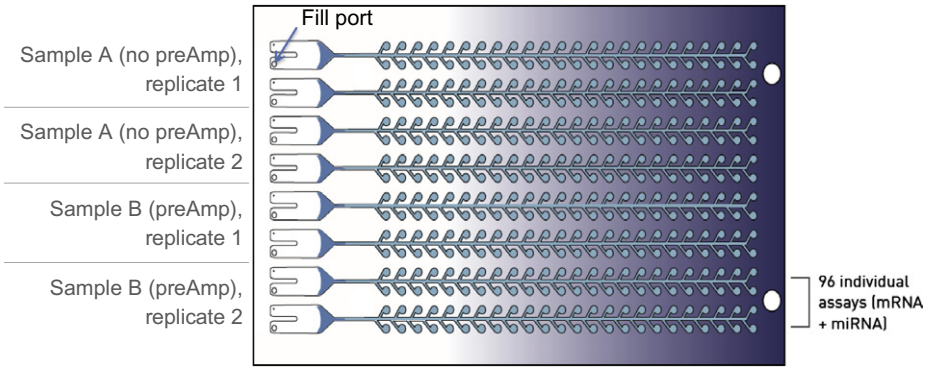

Fig. 1. Experimental setup for the TaqMan® Array Cards. Total RNA was extracted from five pancreatic cancer cell lines and pooled. Reverse transcription was performed without (Sample A) and with (Sample B) a subsequent preamplification step. Expression of the mRNAs and miRNAs was then analyzed using TaqMan® Array Cards containing 79 TaqMan Gene Expression Assays and 6 endogenous control assays and 9 TaqMan miRNA Assays and 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. This gave 4 × 96 data points for both sample A and sample B.

- Identical samples run on two different days cluster very closely, as well (true for both samples A and B, see Fig. 2, samples 1 + 2 vs 3 + 4).
- Gene expression data from the RNA pools (both without and with preamp) cluster closer to each other (i.e., are more similar to each other) than they do to those arising from the tissue samples.
- The four tissue samples fall into two groups: three of the tissue samples are quite similar to each other, while the fourth (solid pseudopapillary tumor; SPT) shows a much lesser degree of similarity, thus accurately 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 preamp samples (5–8) compared to the no preamp samples (1–4). Unfortunately (but not unexpectedly), some of the miRNA candidates are not detected in the RNA pools from cultured cell lines (e.g., miRNA X and miRNA Y, Fig. 2). The tissues were chosen as positive controls, especially for the detection of the miRNAs.

4. Statistical analysis

In order to analyze the reproducibility of the replicates in more detail and to investigate whether a bias is introduced by using the preamp method we applied the "negtrend" statistical method, a method that identifies negligible trends [4], using the exported C_t values as input data.

4.1. Reproducibility—same sample on two days

The reproducibility of identical RNA samples (i.e., comparison of sample A with sample A and sample B with sample B), run on 2 different days, was assessed for mRNA only. For each analyzed gene, five ΔC_t 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, 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".

	Algorithm calls			Percent True*
	True	False	NA	
Sample A (no preamp)	39	22	18	64%
Sample B (preamp)	53	21	6	72%

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 two different days.

The results from sample B (which included a preamp step) show a much higher number of detected genes and a much lower "NA" rate than those of sample A (no preamp step). This demonstrates that including a preamp step leads to more reproducibility and therefore much more reliable data analysis.

4.2. Reproducibility—with and without preamp step

The negtrend algorithm was applied to assess all the combined results from both sample A and sample B (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 True = 57, False = 18 and NA = 4, and for 76% of all detected genes analyzed in this experiment. This means that although a preamp step was performed, for 76% of all detected genes the relative expression results were very similar (TRUE) and that no bias was introduced in 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.

5. Results of preamplification validation

The experiments to validate the preamplification step produced very encouraging results. The workflow gave reproducible results when the same sample was assayed on two different days, and including a preamplification step resulted in a higher number of the genes interrogated giving a True value according to the algorithm used in the analysis. In addition, for a great majority of the genes tested, no bias was introduced when a preamplification step was used. For many of the genes that gave a False value according to the algorithm, the C_t values in the no preamp condition were very high or one of the replicates failed to give a signal, which indicates that those False values weren't a direct result of a failing of the methodology per se. Taken together, these data indicate that

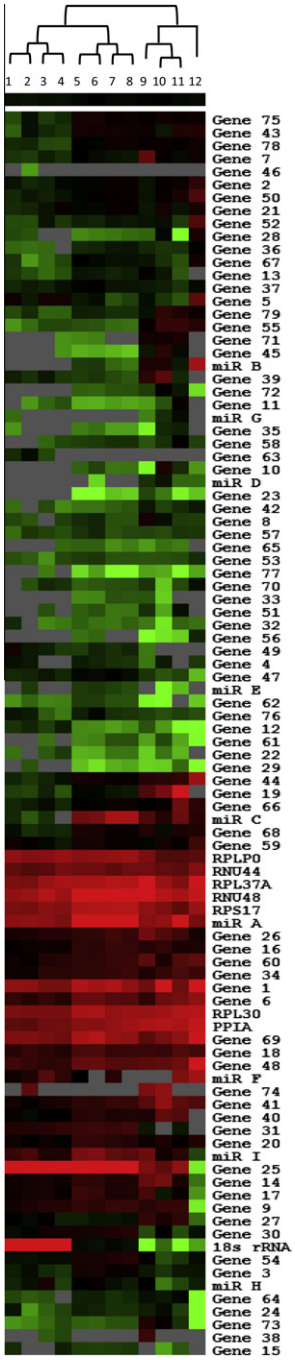

Fig. 2. Hierarchical clustering analysis of expression data for RNA samples across 79 mRNAs and 9 miRNAs. Relative expression values (2–ΔCt) of replicates from Sample A (1–4, no preamplification), Sample B (5–8, preamplification), and the 4 pancreatic tumor samples (9, intraductal papillary mucinous neoplasm, IPMN; 10, pancreatic ductal adenocarcinoma, PDA; 11, chronic pancreatitis; 12: solid pseudopapillary tumor, SPT) were mean-centered and heat maps generated. Group 1, technical replicates; Group 2, the same samples run on different days. Green: low expressed; red: high expressed; gray: not detected.

the preamplification workflow itself is valid and that incorporation of a preamplification step offers significant improvement in the numbers of mRNAs and miRNAs detected.

6. Analysis of FNABs from malignant pancreatic tissues

After successful technical validation of the preamplification workflow described above, a set of 32 FNAB samples 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 procedure and avoid sampling error, and facilitated accurate reference analysis by histopathological evaluation of the resection specimen.

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 A, below.

Each cDNA was analyzed using 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 one gene that was chosen as the "control" gene, and ΔC_t values were calculated. Relative expression values ($2^{-\Delta Ct}$) were plotted and compared. Unsupervised two-dimensional hierarchical cluster analysis of relative gene expression data ($2^{-\Delta Ct}$ method) reveals distinct clusters of pancreatic cancer samples and normal control samples (data not shown).

7. Conclusion

This publication demonstrates the preliminary success of a research method developed for the simultaneous detection of pancreatic cancer-related mRNA and miRNA on TaqMan® Array Cards. Included in the method are the steps to perform simultaneous reverse transcription and preamplification of the targeted genes and a validation procedure to show that bias was not introduced during real-time PCR for the majority of analyzed genes.

The mixed TaqMan® Array Card method has proven to give reliable results and is very easy to use. Dr. Buchholz concludes: "The performance of the "mixed TaqMan Array Cards" has been excellent with respect to reproducibility, sensitivity and specificity. More work needs to be done but the preliminary results obtained by real-time PCR with the cards look very promising. In particular, the combination of mRNA and miRNA gene analysis in a single card represents a tremendous progress in our experimental approach. Our next step is to run the cards with a large set of biopsy samples with known patient history and histological information. We hope to achieve similar or even better accuracy as with our previously developed cDNA Array".

For research use only. Not for use in Diagnostic Procedures.

Acknowledgments

The authors greatly appreciate the opportunity to work with Prof. Thomas Gress and Dr Malte Buchholz from the Clinic for Gastroenterology at the University of Marburg, Germany.

This collaboration was lead by Astrid Ferlinz, FALCON Team, Europe, Life Technologies.

Appendix A. Experimental method

A.1. Step 1: cDNA synthesis

Materials needed

- Custom MicroRNA RT Primer Mix.
- Pooled mRNA preamp Primer Mix.
- Total RNA (1–250 ng).
- TaqMan® microRNA Reverse Transcription Kit (Cat. No. 4366597).
- $MgCl_2$ (Cat. No. AM9530G).

A.1.1. Preparing the RT reactions

Prepare the RT reaction mix in a sterile tube or 96-well plate. 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.

A.1.2. RT reaction mix

Component	Stock	Final	Volume for one reaction (μL)	Volume needed in 20-reaction mix (μL)*
dNTPs with dTTP	25 mM each	0.5 mM	0.20	4.8
MultiScribe™ Reverse Transcriptase	50 U/μL	10 U/μL	2.00	48
10× RT Buffer	10×	1×	1.00	24
1M $MgCl_2$	1M	3 mM	0.03	0.7
AB RNase Inhibitor	20 U/μL	0.25 U/μL	0.13	3.0
H_2O			0.15	3.0

*Volumes listed include ~20% excess.

1. Aliquot 3.5 μL RT reaction mix per well or tube (prepared according to the table above).
2. Add 2 μL of MicroRNA Custom RT Primer Mix.
3. Add 2 μL mRNA Custom Preamp Primer Mix.
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.

A.1.3. Thermal cycling protocol

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

The reverse transcribed (RT) product is stable at −20 °C for at least a week.

A.2. Step 2: Preamplification reaction

Materials needed

- TaqMan® PreAmp Master Mix (Cat. No. 4384266).
- Pooled preamp mRNA/miRNA Primer Mix.
- H_2O.

A.2.1. Preparing the preamplification reactions

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

A.2.2. Preamp primers and enzyme mix

Component	Stock	Final	Volume for one reaction (μL)	Volume needed in 20-reaction mix* (μL)
TaqMan® PreAmp Master Mix (2×)			12.5	300
Pooled preamp mRNA/miRNA Primer Mix	2	05	6.25	150
H_2O			1.25	30

*Volumes listed include ~12.5% excess.

- Aliquot 20 μL preamp primers and enzyme mix per well or tube.
- Add 5 μL of RT product (from step 1) per well/tube.
- Seal tube or plate, mix thoroughly by inverting the plate or tube six times, and then spin briefly.

A.2.3. 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

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

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

Component	Volume needed for one sample on configuration 96a* (μL)
Preamp product	2.25
TaqMan® Universal PCR Master Mix, No AmpErase UNG (Cat. No. 4324018)	112.5
H_2O	110.25
Total volume	225.0*

*Volumes listed include ~12.5% excess.

1. Mix thoroughly and spin briefly.
2. Aliquot 100 μL per port in a TaqMan® Array Card, seal, and spin.
3. *Note:* proper sealing of the TaqMan® Array Card is critical for successful use. See the TaqMan® Array Micro Fluidic Cards User Guide online (search for Part Number 4400263 at lifetechnologies.com).
4. Perform real-time PCR on 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/60sec] for 40 cycles).

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