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Optimized protocol with low sample input for profiling human microRNA using the OpenArray® platform

The OpenArray® system provides a highly efficient platform for profiling human and rodent microRNAs (miRNAs). Using Megaplex™ RT primer pools and PreAmp primer pools for TaqMan® MicroRNA Assays, the system can profile three samples on a single OpenArray® plate. This means that in one full run with three OpenArray® plates, you can profile nine individual samples for 754 human miRNAs (excluding controls) in just under 3 hours (typical run time), and in a single day you can generate 27,144 raw data points with a total of 36 samples over four runs. This makes the OpenArray® platform a valuable solution for high-throughput miRNA profiling.

The current Applied Biosystems® OpenArray® system protocol recommends 100 ng of total RNA sample input for each of the two Megaplex™ RT primer pools. However, in certain situations where RNA yield is limited, such as with laser capture microdissection or needle-aspiration biopsies, allowing reduced RNA input is desirable. We have developed a Low Sample Input (LSI) protocol for these types of reduced-yield samples. To validate the profiling performance of this LSI protocol, we tested both 10 ng and 1 ng of total RNA per pool, and compared the tissue-to-tissue differential expression data with those obtained with the standard protocol (with 100 ng of total RNA input).

Table 1. Reverse transcription reaction setup.

Component	Stock	Final (1X)	Volume (µL)
Total RNA	2.5X	1X	3.00
Megaplex™ RT Primers (10X), **Pool A or Pool B**	10X	1X	0.75
dNTPs with dTTP (100 mM)	50X	1X	0.15
MultiScribe™ Reverse Transcriptase	50 U/µL	10 U/µL	1.50
10X RT Buffer	10X	1X	0.75
$MgCl_2$ (25 mM)	25 mM	3 mM	0.90
RNase Inhibitor	20 U/µL	0.25 U/µL	0.09
Nuclease-free water			0.36
		Total (µL)	7.50

Materials and methods

We used 100 ng, 10 ng, and 1 ng total RNA from human brain (Applied Biosystems® P/N AM7962) and lung (Applied Biosystems® P/N AM7968), per Megaplex™ RT primer pool, to model tissue-to-tissue differential expression of miRNAs. Megaplex™ RT Primers, Human Pool A v2.1 (Applied Biosystems® P/N 4399966) and Megaplex™ RT Primers, Human Pool B v3.0 (Applied Biosystems® P/N 4444281) were used for reverse transcription. Megaplex™ PreAmp Primers, Human Pool A v2.1 (Applied Biosystems® P/N 4399233) and Megaplex™ PreAmp Primers, Human Pool B v3.0 (Applied Biosystems® P/N 4444303) were used for preamplification. The TaqMan® OpenArray® Human MicroRNA Panel (Applied Biosystems® P/N 4461104) was used for final PCR detection.

Workflow

All sample input amounts were processed according to a full standard protocol. The data from the 100 ng sample input were used as reference for profiling, and the data from the 10 ng and 1 ng sample inputs were used as a baseline for optimization. Replicate lower-input

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samples (10 ng and 1 ng) were processed using the LSI protocol that starts with a standard reverse transcription (RT) reaction as shown in Table 1.

Separate preamplification (PreAmp) mixes were created for pools A and B, according to Table 2. In the same 96-well plate used for RT, 32.5 µL PreAmp mix for the appropriate pool was added to the RT product. The final volume for the PreAmp reaction was 40 µL. Samples were cycled with a standard PreAmp thermal protocol for 16 cycles. By performing RT and PreAmp in the same 96-well plate, the LSI delivers a more streamlined workflow.

The PreAmp product was diluted 1:20 using 0.1X TE. Then samples were loaded onto OpenArray® plates and cycled according to the standard protocol.

Data analysis

Detection rate

One of the most important considerations for profiling experiments is the number of miRNAs that can be detected. With the standard protocol, the number of miRNAs detected drops dramatically as the input amount is reduced to 10 ng and to 1 ng (Figure 1 and Table 3). In contrast, use of the LSI protocol with 10 ng of input resulted in the recapture of 49 miRNAs. (The recaptured miRNAs were defined by Cq >25 in at least one of the two tissues tested using the standard protocol, whereas Cq became <25 in both tissues after using the LSI protocol.)

The ability to capture more miRNAs extends even to the lowest input amount tested, 1 ng. The number of miRNAs increased from 70 (standard protocol) to 125 (LSI protocol). In fact, the number of miRNAs detected with 1 ng of sample input using the LSI protocol is 93% of the number of miRNAs detected with 10 ng of sample input using the standard protocol (125 vs. 135).

Table 2. Megaplex™ PreAmp reaction setup.

Component	Stock	Final (1X)	Volume (µL)
RT product	1X	1X	7.5
PreAmp mix			
TaqMan® PreAmp Master Mix (2X)	2X	1X	20.0
Megaplex™ PreAmp Primer Mix (10X), **Pool A or Pool B**	10X	1X	4.0
Nuclease-free water			8.5
		Total (µL)	40.0

Table 3. Total number of miRNAs co-detected in both brain and lung. The result obtained with a sample input of 100 ng is used as the reference (100%). Std: standard protocol; LSI: low sample input protocol.

Input	100 ng-Std	10 ng-Std	10 ng-LSI	1 ng-Std	1 ng-LSI
Pool A + B	201	135	184	70	125
%	100%	67%	92%	35%	62%

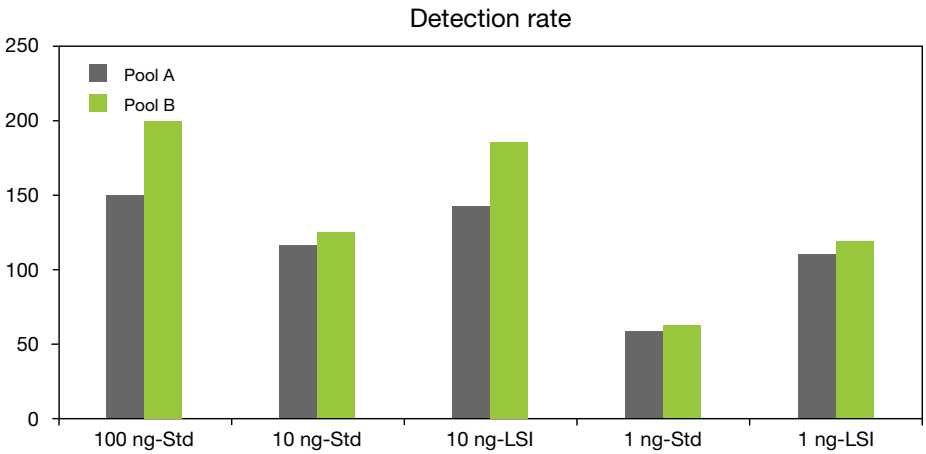

Figure 1. Number of miRNAs co-detected in both brain and lung. Only Cq values that were less than the Cq cutoff were graphed. The numbers of assays from pool A are represented in gray, and from pool B in green. The results obtained with a sample input of 100 ng are the reference (far left). Std: standard protocol; LSI: low sample input protocol.

Quantification cycle (Cq) of recaptured miRNAs

The Cq values of all detected miRNAs were graphed in histogram form without the Cq cutoff at 25 (Figure 2). The 49 recaptured miRNAs are highlighted in darker green. For the lung sample, using the standard protocol and 10 ng of input material, most of these 49 miRNAs had Cq values higher than the cutoff (Figure 2E). With the LSI protocol, all of them were less than the cutoff value (Figure 2F).

When evaluating the distribution of the Cq values of the recaptured 49 miRNAs, we observed that the LSI protocol was able to return Cq values and distributions that were similar for both tissues and were comparable to the reference (100 ng sample input with the standard protocol) (Figures 2A and 2D vs. Figures 2B, 2C, 2E, and 2F).

Concordance with normal sample input and standard protocol

For the next stage of data analysis, we evaluated the profiling data of the recaptured 49 miRNAs (obtained with the LSI protocol and 10 ng sample input) by ΔΔCq (see Appendix for definition) concordance against data from 100 ng sample inputs with the standard protocol. These results will be an indication

of how well the LSI protocol performs, compared to the standard protocol, on miRNAs that are more differentially expressed (high |ΔΔCq|) or similarly expressed (low |ΔΔCq|) between two tissues.

As shown in Figure 2, ΔΔCq from LSI protocol with 10 ng of sample input exhibited a high level of concordance with data obtained from the standard protocol with 100 ng of sample input (Figure 3, right panel, R^2 = 0.933). In addition, the slope of ΔΔCq correlation is improved from 0.945 (standard protocol) to 0.984 (LSI protocol).

The 49 recaptured miRNAs are shown as red dots in Figure 3 (right panel) and their ΔΔCq values are distributed evenly along both sides of the regression line between –6 and 8, indicating that the 49 miRNAs are captured by the LSI protocol independently of their tissue expression levels. In a separate experiment, we spiked five 10-fold dilutions of a nonhuman miRNA into 10 ng of total RNA from either brain or lung as background, using the LSI protocol. We were able to detect it at all five concentrations with excellent linearity, with R^2 larger than 0.995 and PCR efficiencies of 100 ± 20%.

ΔΔΔCq Distribution

We examined the distribution of ΔΔΔCq (see Appendix for definition) for the miRNAs detected by the standard and LSI protocols. ΔΔΔCq allows us to compare the magnitude of the ΔΔCq change when 10 ng of sample input is processed by the standard and LSI protocols; a small ΔΔΔCq indicates a similar expression pattern with 10 ng input as with 100 ng input.

As shown in Figure 4, the LSI protocol returns a distribution of ΔΔΔCq that is comparable to the standard protocol for 10 ng of sample input and centered on 0. Most of the 49 recaptured miRNAs have ΔΔCq between 1 and –1, and have a similar distribution pattern compared to the overall population (dark green segments in Figure 4, right panel), further confirming that the

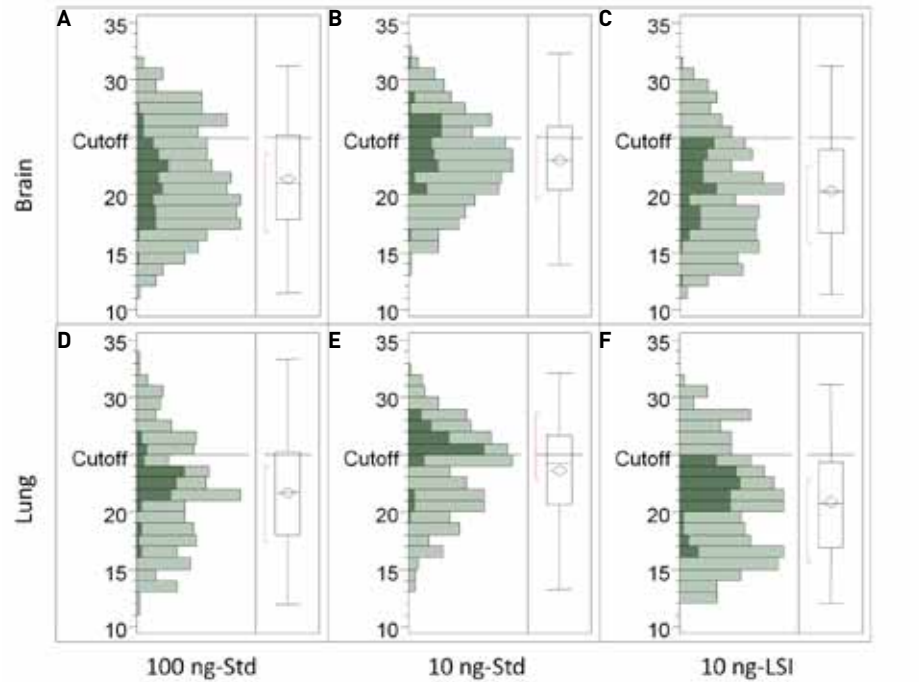

Figure 2. Distribution of Cq values. Histograms of Cq values obtained from both brain and lung are plotted without the Cq cutoff at 25 (sample input and protocol are indicated at the bottom). The 49 recaptured assays are highlighted in darker green. Std: standard protocol; LSI: low sample input protocol.

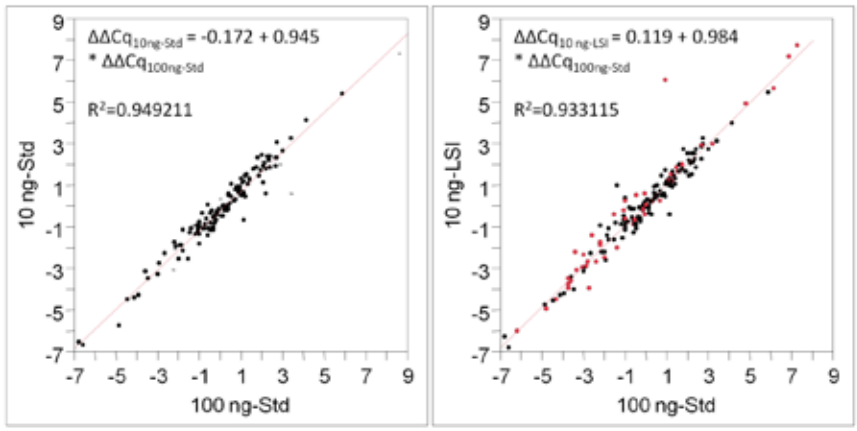

Figure 3. Concordance of ΔΔCq. The axis labels indicate the sample input and protocol from which the ΔΔCq is derived. The linear regression and coefficient of determination R^2 are shown . Red dots represent miRNAs detected by the LSI but not the standard protocol with 10 ng of sample input. Std: standard protocol; LSI: low sample input protocol.

LSI protocol rescued the 49 miRNAs but did not skew their expression patterns compared to those obtained using the standard sample input and protocol.

Background noise

Background noise was determined by the number of assays that had a Cq value less than the cutoff value in the no-template control (NTC) (Figure 5); all the miRNAs we detected in the two tissues had Cq values less than those obtained from the NTC.

Conclusion

In certain situations where the RNA yield cannot accommodate our recommended 100 ng RNA per pool of input, we provide an optimized protocol using the entire RT product for the preamplification step (16 cycles), and 1:20 post-preamplification dilutions, with 10 ng of total RNA per pool. This LSI protocol allows us to achieve a higher detection rate without sacrificing profiling performance—in an assay that is not affected by background noise. If there is even less than 10 ng of input sample available, increasing the number of preamplification cycles and/or reducing the post-preamplification dilution factor are options, but these might be limited in benefit, and caution must be taken to interpret data from false positive signals and loss of linearity.

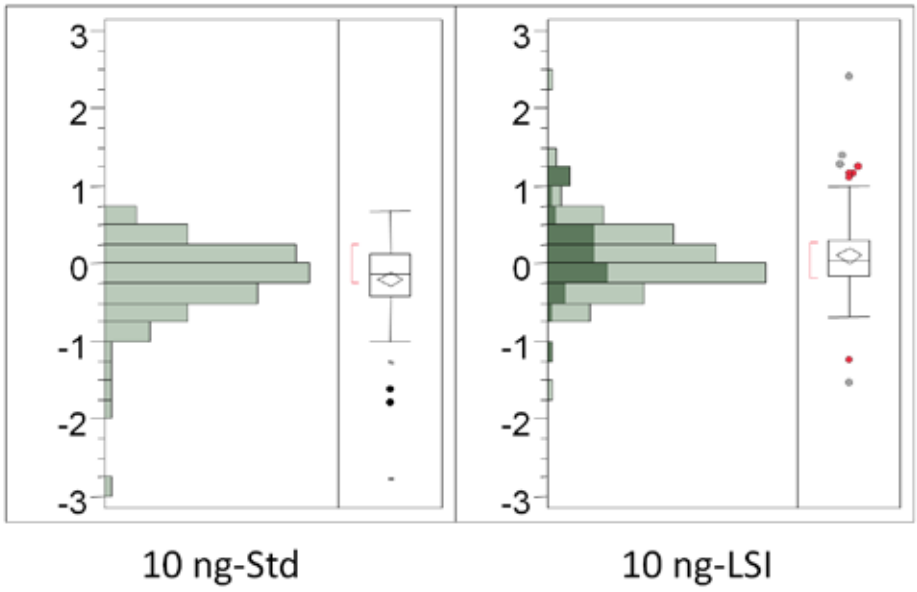

Figure 4. Distribution of ΔΔΔCq from standard or LSI protocol with 10 ng of sample input. Assays that are co-detected between 10 ng sample input of either protocol and 100 ng sample input were used for plotting. Areas highlighted with darker green indicated miRNAs that are uniquely detected with the LSI but not the standard protocol with 10 ng of sample input. Std: standard protocol; LSI: low sample input protocol.

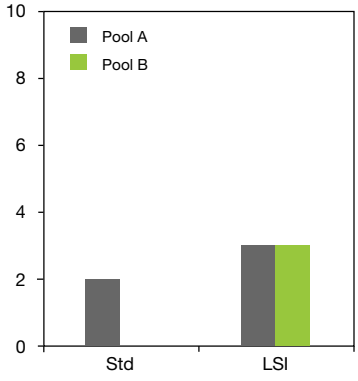

Figure 5. Background noise determined by the number of assays showing Cq values less than cutoff when NTC was used. Std: standard protocol; LSI: low sample input protocol.

Appendix: miRNA relative quantification method

$$\Delta Cq = Cq_{\text{miRNA of interest}} - Cq_{\text{global mean}}$$
$$\Delta\Delta Cq = \Delta Cq_{\text{brain}} - \Delta Cq_{\text{lung}}$$
$$\Delta\Delta\Delta Cq = \Delta\Delta Cq_{\text{10 ng-protocol}} - \Delta\Delta Cq_{\text{100 ng-Std}}$$

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