Optimized protocols for human or rodent microRNA profiling with precious samples

Life Technologies' Megaplex™ Primer Pools with matching TaqMan® Array MicroRNA Cards or OpenArray® MicroRNA Panels are widely used for microRNA (miRNA) expression profiling. The pools, along with their matching cards or plates, are designed to quantitate >750 miRNAs (381 per pool) in human, mouse, or rat species using stem-loop reverse transcription (RT) primers and TaqMan® Assay chemistry. Increasingly, researchers are requiring better assay sensitivity with lower sample input amounts (<1 ng) for their profiling studies. The standard Megaplex™ Primer Pools protocol is amenable to further optimization to enhance detection under challenging conditions for these precious samples. In this technical note, we give general optimization guidelines for profiling using limited sample input with TaqMan® Array MicroRNA Cards. The same approach can be used for any sample type—e.g., serum, plasma, or formalin-fixed, paraffin-embedded (FFPE)—run on TaqMan® Array Cards or OpenArray® Panels.

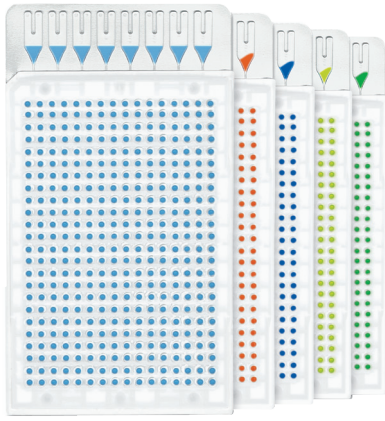

Table 1. MicroRNA profiling with Megaplex™ Primer Pools and corresponding TaqMan® MicroRNA Cards and OpenArray® MicroRNA Panels.

Species	Megaplex™ Pool Set	Cat. No.	TaqMan® Array Card Set	Cat. No.	TaqMan® OpenArray® MicroRNA Panel	Cat. No.
Human	Human Pool A + B, RT + PreAmp Primers Set v3.0	4444750	Human Card A + B Set v3.0	4444913	Human Panel	4470187
Rodent	Rodent Pool A + B, RT + PreAmp Primers Set v3.0	4444766	Rodent Card A + B Set v3.0	4444909	Rodent Panel	4470188

Sets of A + B, RT primer pools and accompanying PreAmp primer pools provide coverage for up to 381 miRNAs per pool in human and rodent (mouse and rat). Each primer pool set is used with a matching human or rodent TaqMan® Array MicroRNA Card Set or a TaqMan® OpenArray® MicroRNA Panel for qPCR readout. TaqMan® Array A and B Cards and Megaplex™ Primer Pools can be purchased individually as well. Go to **lifetechnologies.com** for more information.

Protocol optimization

The standard Megaplex™ Primer Pools protocol for analysis using TaqMan® Array Cards consists of a reverse transcription (RT) reaction, an optional preamplification step, and a final quantitative PCR (qPCR) run on a TaqMan® Array MicroRNA Card (see the *Megaplex™ Pools for microRNA Expression Analysis* protocol, PN4399721). To optimize the protocol for achieving the highest detection rates, we focused on the preamplification step and assessed four alternative (modified) protocols, each with at least two modifications from the standard (Table 2):

1. Reduce the total dilution of the preamplification product in qPCR from 1:400 to 1:200 or 1:100;
2. Increase the number of preamplification cycles from 12 to 14;
3. Change the dilution of the RT reaction in the preamplification reaction from 1:10 to 1:5.

Protocol	RT dilution	# of PreAmp cycles	PreAmp dilution
Standard	1:10	12	1:400
Modified I	1:5	12	1:200
Modified II	1:5	12	1:100
Modified III	1:5	14	1:200
Modified IV	1:5	14	1:100

Table 2. Protocol optimization using standard and modified conditions.

Materials and methods

The optimization was performed using the Megaplex™ Primer Pools, Human Pools Set v3.0 and matching TaqMan® Array MicroRNA Cards set. We chose total RNA samples from two normal human tissues, brain and lung (FirstChoice® Total RNA). All RT and preamplification reactions were performed according to the standard protocol except for their final reaction volumes, which were 5 µL and 25 µL, respectively. Each sample (25 ng, 1.0 ng, or 0.1 ng total RNA) was run separately with each pool. TaqMan® Array MicroRNA Cards were run in duplicate per condition on an Applied Biosystems® 7900HT Fast Real-Time PCR System and analyzed with SDS 2.4 software. The final optimized protocol was used to re-run the samples in triplicates to confirm the data.

Data analysis

Determining threshold cycle (C_t) cutoff

With TaqMan® Array Cards, average C_t values >32 for cDNA samples are often considered to be below the limit of reliable detection. Thus, C_t values <30 on an array card with amplified sample are positive reactions reflecting detectable cDNA target copies in the sample. C_t values between 30 and 32 are positive, but reflect low amounts of target; C_t values between 32 and 40 reflect very low copy numbers (single-digit to null). The confidence in data for low-expressing genes improves with either an increase in number of replicates for each sample, or a decrease in the standard deviation of the C_t of the replicates. Thus, the first step in the data analysis includes evaluating the variability of the data set and identifying the C_t threshold to determine reliable data. To identify the cutoff we evaluated the standard deviation (SD) of the C_t with increasing C_t values. Setting the upper limit of acceptable SD of the C_t at 0.30, we selected a cutoff of C_t = 30 for preamplification using 12 cycles, and a cutoff of C_t = 29 for 14 cycles (Table 3). All subsequent data analysis results were obtained using these cutoff values.

Table 3. Average standard deviations for each unit C_t interval. Recommended cutoffs for each tested condition based on the SD for C_ts.

	Recommended C_t cutoff	Avg SD
Standard	30.0	0.23
Modified I	30.0	0.24
Modified II	30.0	0.23
Modified III	29.0	0.24
Modified IV	29.0	0.20

Criteria for determining optimal conditions

We used 25 ng of total RNA in the RT reaction as the standard condition. The result from each modified condition was compared to the standard. We evaluated the following criteria to determine the condition that gave the best performance:

1. Highest detection rate—the greatest number of assays with C_t below the cutoff
2. Best concordance (R^2) with the 25 ng standard condition
3. Lowest background noise—the lowest number of assays in the no-template control (NTC) reaction that show C_t values below the cutoff

Detection rate

The driving criterion for optimization is to maximize the detection rate. As expected, detection rate decreases with decreasing amounts of total input RNA. Likewise, the number of detectable miRNAs increases as the dilution factor is reduced and the number of preamplification cycles is increased (Figure 1). For 1 ng of sample input, the highest detection rate is obtained with the lowest dilution factor (1:100) and the highest number of preamplification cycles (14).

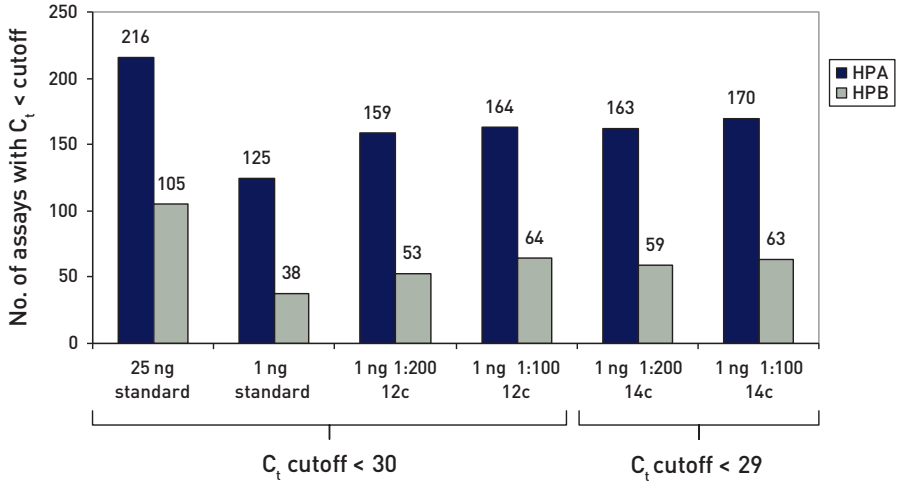

Figure 1. Optimization of conditions for detection of miRNAs. Numbers of assays shown are averages from triplicate TaqMan® Array Card runs with brain and lung samples. Sample amounts, nonstandard dilution factors, and number of preamplification cycles (12 or 14 cycles) are specified. HPA: Human Pool A; HPB: Human Pool B.

Concordance with standard condition

We analyzed concordance between the standard and modified protocols using two separate parameters: correlation (R^2) of the $\Delta\Delta C_t$, and percentage of $\Delta\Delta\Delta C_t$ values < 1.0.

The $\Delta\Delta C_t$ value is determined by the difference between ΔC_t values from two samples. As a standard, fold change is determined from the formula $2^{-\Delta\Delta C_t}$:

$\Delta C_t = C_t$ (assay) – C_t (U6 snRNA endogenous control)
$\Delta\Delta C_t = \Delta C_t$ (brain) – ΔC_t (lung)

The $\Delta\Delta\Delta C_t$ value is determined by the difference between the $\Delta\Delta C_t$ of the standard protocol and modified protocol:

$\Delta\Delta\Delta C_t = \Delta\Delta C_t$ (25 ng standard protocol) – $\Delta\Delta C_t$ (1 ng modified protocol)

$\Delta\Delta\Delta C_t$ < 1.0 (less than 2-fold change) indicates that there is little difference between the two conditions and that they produce similar expression patterns.

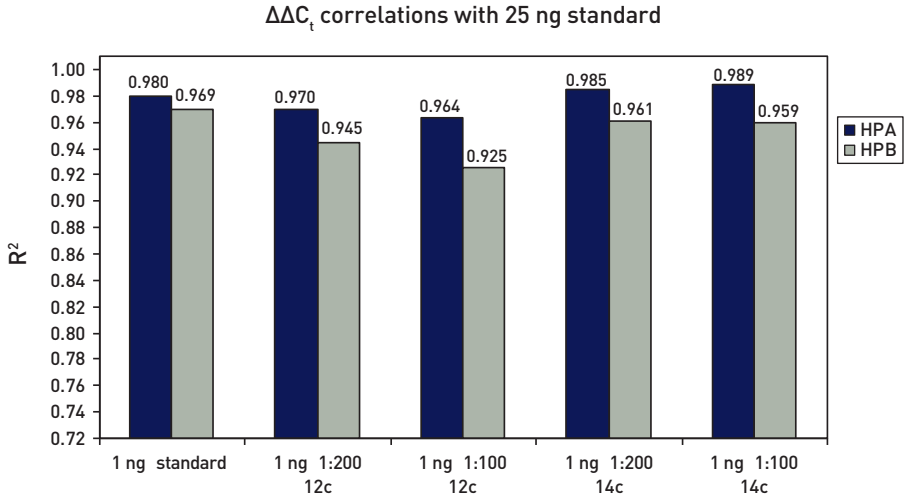

Figure 2. ΔΔC$_t$ correlations with 25 ng standard. Linear regression analysis for the ΔΔC$_t$ was performed, including only assays that were positive in both the 25 ng standard and the modified protocols. Correlation coefficients (R^2) are shown. For ΔΔC$_t$, the 1:200 dilution with 14 preamplification cycles and 1:100 dilution with 14 cycles show the highest correlations in both Human Pool A (HPA) and Human Pool B (HPB). The 1 ng sample run under standard conditions is shown for reference.

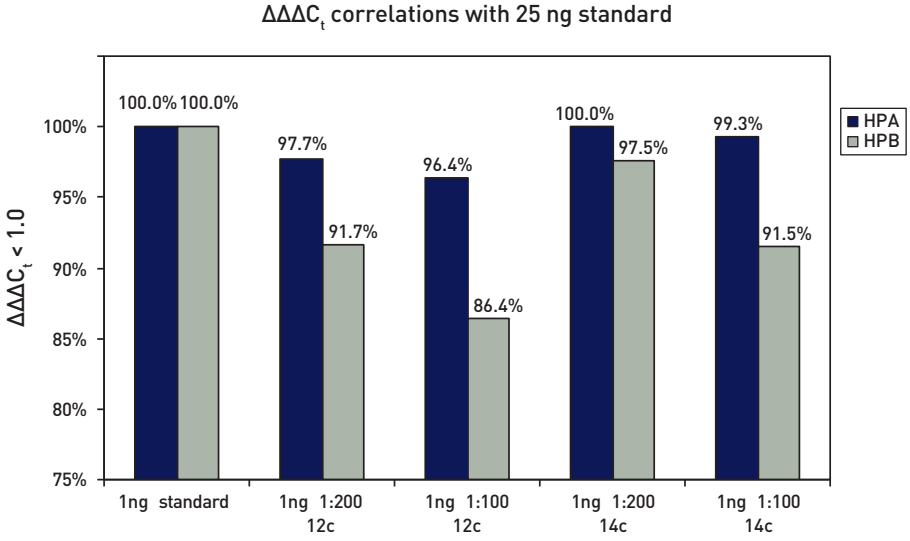

Figure 3. ΔΔΔC$_t$ correlations with 25 ng standard. ΔΔΔC$_t$ was determined for assays that were positive for both the 25 ng standard and the modified protocols. The percentages of assays with ΔΔΔCt < 1.0 are shown for Human Pool A (HPA) and Human Pool B (HPB). The highest correlation in both pools A and B is seen with the 1:200 dilution and 14 cycles of preamplification. The 1 ng standard is shown for reference.

For the ΔΔC$_t$ R^2 analysis, the modified protocols with 14 preamplification cycles had better correlation to the 25 ng standard than 12 preamplification cycles with either dilution (Figure 2). Similarly, the ΔΔΔC$_t$ analysis showed that the highest percentage of assays showing expression patterns similar to the standard was obtained with 14 preamplification cycles and 1:200 dilution. Under these conditions, 100% and 97.5% of detectable assays on TaqMan® Array Cards A and B, respectively, had ΔΔΔC$_t$ < 1.

Background noise

Background noise due to nonspecific primer interactions was determined by counting the number of assays with signal in the NTC reactions where the C_t was below the respective cutoff for each condition (Table 3). The least background signal is seen with the 1:200 dilution and 14 preamplification cycles (Figure 4).

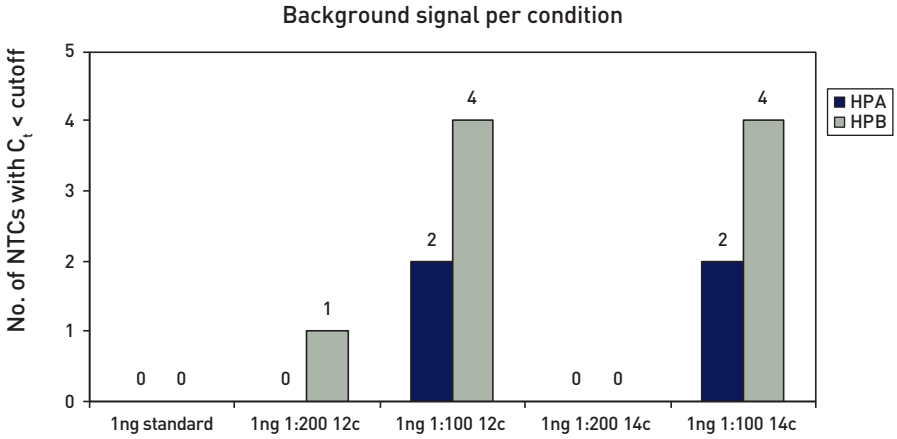

Figure 4. Nonspecific background signal per condition. Background signal was determined by the number of assays in the NTC reaction showing C_t below the cutoff for the NTC reaction. HPA: Human Pool A; HPB: Human Pool B.

Selection of optimal conditions

From these data, we determined that the conditions that simultaneously provided the highest detection rate, the best correlation compared to the 25 ng standard protocol, and the lowest background consist of:

- Reducing the RT reaction volume to 5 µL and the RT-to-preamplification dilution ratio to 1:5
- Increasing the number of preamplification cycles to 14
- Diluting the preamplification products 1:200 before loading the matching TaqMan® Array Card

Further statistical analysis using JMP 8.2 statistical software confirmed that these were the best conditions for meeting the stated criteria (data not shown).

Detection of low sample input

Having determined the optimal protocol for maximized detection rate, we examined this condition on a 10-fold lower sample input (0.1 ng) and assessed the results. When compared to the standard protocol with 1 ng input, the optimized protocol with 1 ng input detects approximately 40% more targets, and the optimized protocol with 0.1 ng input detects approximately 70% of the targets (Figure 5A). Strong correlation for Pool A (100%) and Pool B (97.6%) is observed with 1 ng input, with a slight compromise with 0.1 ng (Figure 5B).

We have therefore shown that a detection rate of approximately 70% is achievable, even with only 0.1 ng of sample input, using the optimized protocol. The concordance of the 0.1 ng data relative to the 25 ng standard, as well as the selection of the appropriate C_t cutoff, provides confidence for reliable detection.

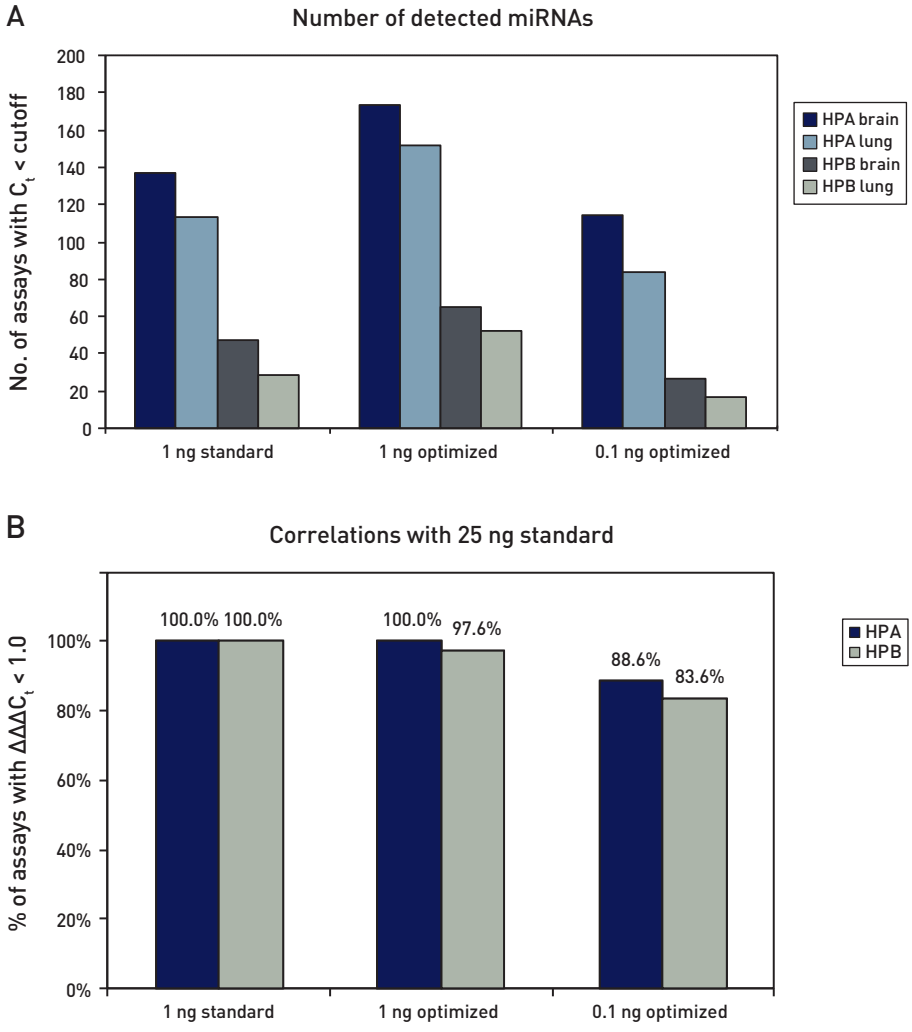

Figure 5. Detection of miRNAs using low sample input. (A) Comparison of miRNA detection across two Megaplex™ Primer Pools (Human Pool A (HPA) vs. Human Pool B (HPB)), two tissues (brain vs. lung total RNA), two protocols (standard vs. optimized), and, for the optimized protocol, two sample input amounts (1 ng vs. 0.1 ng) with n = 3. **(B)** Correlation of gene expression of the 1 ng and 0.1 ng input using the optimized protocol compared to the standard protocol (brain and lung total RNA).

Discussion

For the Megaplex™ Primer Pools protocol optimization, the interplay between three parameters was considered: detection rate, concordance with the standard protocol, and background noise detected in no-template controls (NTC). We used this approach to develop a modified protocol for low input amount or precious samples (1 ng or lower, such as FFPE). The modifications included reducing the RT reaction volume, using 14 preamplification cycles instead of 12, and reducing the final dilution ratio of the preamplification product in the final qPCR reaction to 1:200. For very low sample amounts, for example, those in the picogram range, or difficult samples, such as blood plasma, a higher detection rate may be more important than a low background or strict concordance. Thus, alternative strategies may be used to capture the most data points. For example, increasing the C_t cutoff value, decreasing the preamplification dilution ratio, or increasing the number of preamplification cycles may provide higher detection rates (data not shown). However, these strategies may pose a drawback in the form of reduced specificity (due to off-target primer hybridization), as well as higher background noise (due to low-level primer interactions that only appear at higher C_ts). A proper balance between sensitivity and specificity may need to be determined to best meet the goals of the user.

A similar approach was used to optimize low sample input (LSI) or blood plasma with Megaplex™ Primer Pools on the OpenArray® platform. In these studies, TaqMan® Array Cards using the LSI protocol presented here were used as the reference for LSI on the OpenArray® instrument. For blood plasma, an optimized protocol was developed in parallel with the OpenArray® platform. The conditions for these types of samples are presented in Table 4.

Table 4. Overview of the steps for the standard, low sample input (LSI), and plasma (and blood products) protocols for both the TaqMan® OpenArray® Panels and for the TaqMan® Array Cards.

	Standard		LSI		Plasma	
	OpenArray® Panels	TaqMan® Array MicroRNA Cards	OpenArray® Panels	TaqMan® Array MicroRNA Cards	OpenArray® Panels	TaqMan® Array MicroRNA Cards
Recommend input amount (per pool)	100 ng	50–350 ng	10 ng	1–10 ng	3 µL	3 µL
RT volume (µL)	7.5	7.5	7.5	5	7.5	10
RT to preamplification volume (µL)	2.5	2.5	7.5	5	7.5	10
Final preamplification volume (µL)	25	25	40	25	40	50
No. of preamplification cycles	12	12	16	14	16	14
Post-preamplification dilution ratio	1:40	1:4	1:20	1:4	1:40	None
Dilution from the PCR reaction ratio	1:2	1:100	1:2	1:50	1:2	1:100
Final dilution ratio	1:80	1:400	1:40	1:200	1:80	1:100

Conclusions

In this technical note we present an optimized Megaplex™ Primer Pools protocol to enhance quantitation when profiling miRNAs on TaqMan® Array MicroRNA Cards under conditions with limited sample input (1 ng total RNA per pool). We have shown that by using all of the RT product volume instead of 2.5 µL, increasing the number of preamplification cycles, and changing the qPCR dilution ratio, the detection rate increases significantly without compromising assay specificity or increasing background signal noise. Furthermore, this optimized protocol allows high detection rates to be maintained with as little as 100 pg of sample.

We have also included a summary of miRNA profiling protocols recommended by Life Technologies for limited sample inputs and for blood plasma samples with TaqMan® Array MicroRNA Card sets or TaqMan® OpenArray® MicroRNA Panels (Table 4). Finally, we provide general guidelines to support further optimization based on users' specific optimization needs and particular sample conditions.

References

- Application Note: *Optimized protocol with low sample input for profiling human microRNA using the OpenArray® platform*
- Application Note: *Optimized blood plasma protocol for profiling human miRNAs using the OpenArray® Real-Time PCR System*