Technical Note

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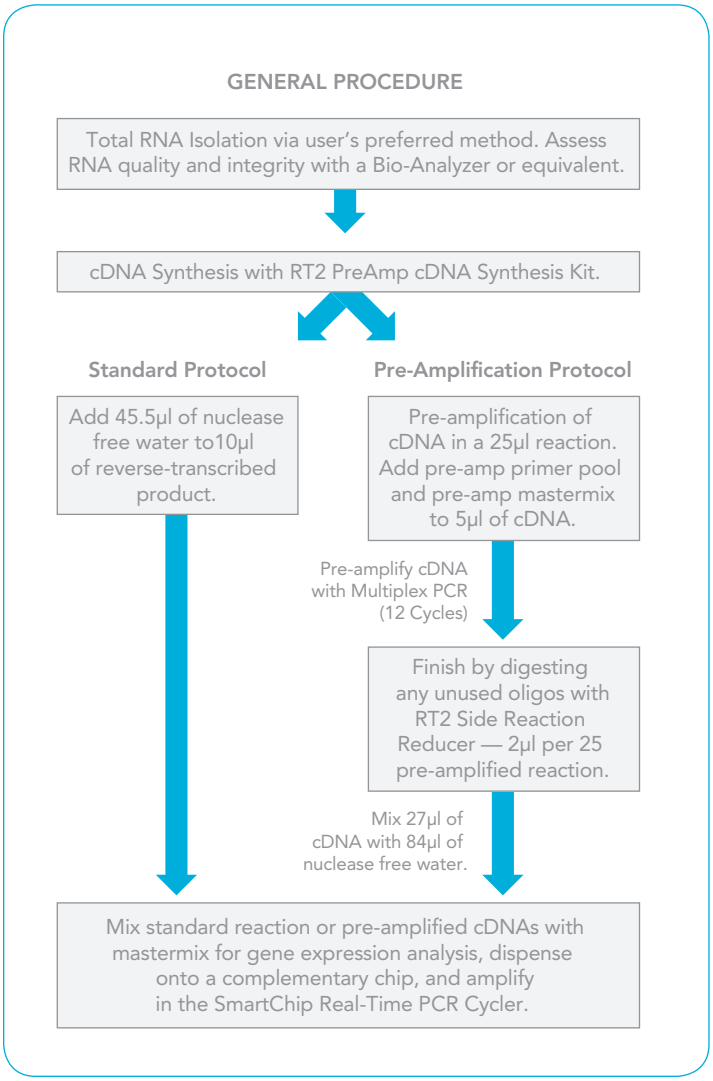
Targeted Enrichment of Limited RNA Samples via Pre-Amplification Prior to Analysis in the WaferGen SmartChip Real-Time PCR System

Summary

Quantitative Polymerase Chain Reaction (qPCR) is the most routinely used and reliable technique for the quantification of gene expression biomarkers. However, as a result of low RNA extractable amounts often obtained from FFPE samples, flow-sorted cells, or reduced starting tissue, gene expression researchers are often faced with samples available in inadequate quantities or conditions to adequately serve as template for qPCR. While in many instances the SmartChip technology can successfully detect low copy transcripts without preamplification, there may be instances for very low transcript levels in which some preamplificaiton is beneficial. In order to overcome this problem, WaferGen Biosystems has adapted a quick and easy multiplex pre-amplification protocol that will allow users to profile a large set of genes when the starting material is scarce.

The multiplex pre-amplification PCR approach has the ability to increase the detection of low abundance biomarkers, as well as to provide the ability to extend the number of analyses that can be completed on a single sample. Pre-amplification involves three general steps: cDNA synthesis from RNA, enrichment of targeted cDNAs via multiplex pre-amplification PCR, and analysis of enriched cDNA library with complementary assays.

The work presented here demonstrates that the enriched cDNA products made with the first two steps previously mentioned is adaptable to the high-throughput WaferGen SmartChip System. The results also demonstrate that an enriched 1ng of RNA produces similar results as 1µg of RNA that was not pre-amplified.



Materials and Methods

The following figure summarizes each of steps taken to synthesize cDNA from total RNA, enrich cDNA via pre-amplification, and analyze the cDNA preparations in

Figure 1. ***Steps taken to analyze samples in the WaferGen Smart Chip System.*** *RNA is first isolated via user's preferred method. Next, the RNA preps are analyzed for their integrity in the Bioanalyzer or Equivalent (Preps with RNA Integrity Number (RIN) of 8 or higher work best). RNA is then converted to cDNA with the RT2 PreAmp cDNA Synthesis Kit. Following cDNA synthesis, concentrations of starting RNA that are higher than 1µg can be processed with the Standard Protocol. Concentrations of RNA that are ≤1ng of RNA are recommended to be processed with the Pre-amplification Protocol.*

the WaferGen Smart Chip System. Each of these steps is described in more detail in the following sections.

RNA Samples

Total RNA samples from human universal reference (UHRR) (P/N 6365238) were obtained from Clontech Laboratories (Mountain View, CA) and Brain reference (Brain) (P/N AM6050) from Life Technologies (Foster City, CA). Both RNA samples were diluted to 200ng/µl, 0.2ng/µl, 0.02ng/µl, and 0.002ng/µl.

cDNA Synthesis

cDNA was synthesized using 5µl from each dilution of UHRR and Brain RNA and the components of the SABiosciences RT2 PreAmp cDNA Synthesis Kit (P/N 330451) (Qiagen, Valencia, CA). Total amount of template RNA per reaction was therefore 1µg, 1ng, 0.1ng, and 0.01ng across the dilution series. All cDNA reactions were completed in a total volume of 20µl. After cDNA synthesis the reactions with 1µg RNA template received the addition of 91µl of nuclease free water but did not undergo pre-amplification. The reactions with 1ng, 0.1ng, and 0.01ng RNA template received no additional water after cDNA synthesis but underwent the pre-amplification procedure.

Multiplex Pre-Amplification

Multiplex PCR pre-amplifications of 96 targets were performed according to SABiosciences recommendations. Briefly, 96 primer sets corresponding to a validated assay panel were pooled together to a final concentration of 0.4µM. Next, 7.5µl of pre-amplification primer pool were mixed with 12.5µl of RT2 PreAmp Mastermix and a total of 5µl of cDNA. The entire solution was mixed gently and quickly centrifuged. All pre-amplification reactions were cycled in a standard thermal cycler using the following parameters: 10 minutes at 95°C, 12 2-step cycles composed of 15 seconds at 95°C and 2 minutes at 60°C, and a 5 minute hold at 4°C. Following pre-amplification, any unused primers were digested with 2µl of Side Reaction Reducer. After the addition of this reagent, each pre-amplified reaction was incubated for 15 minutes at 37°C and followed by 5 minutes at 95°C for any enzyme activity inactivation. Immediately after enzyme inactivation, 84µl of nuclease free water was added to each reaction.

qPCR Amplifications and Arrays

Since the SmartChip MyDesign Chip is easily configurable, the 96 primer pairs previously used for pre-amplification were individually dispensed onto a MyDesign Chip. For each sample, each assay was dispensed in quadruplicates. The chip was treated per WaferGen Biosystems recommendations then cycled in the SmartChip Cycler using the following thermal parameters: 10 minutes at 95°C, 45 2-step cycles composed of 37 seconds at 95°C and 70 seconds at 60°C. Immediately following amplification, melt curve analysis was performed from 60°C to 97°C.

Results

The delta Ct threshold cycle (dCt) values for the entire array containing a subset of 96 assays were compared and analyzed. The resulting scatter plot comparison between the 1µg of RNA processed with the Standard Protocol and the 1ng of pre-amplified RNA yield a straight line with a slope of 0.96 and correlation

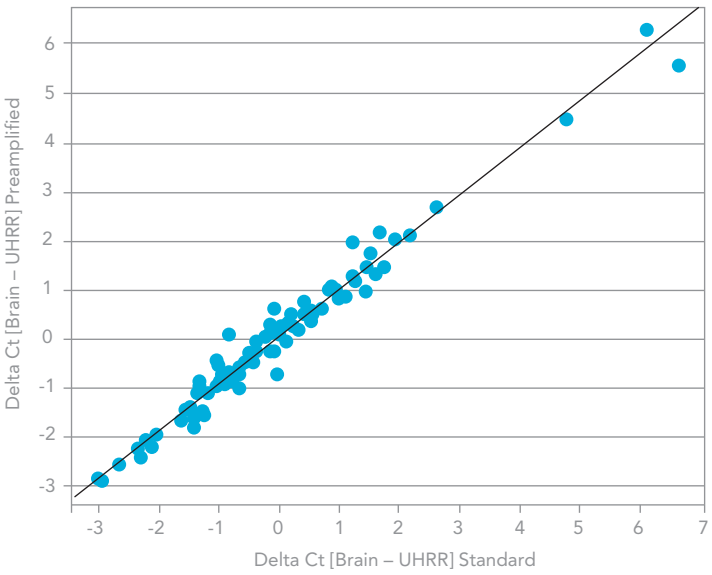


Figure 2. ***Comparison between 1µg of starting RNA and 1ng enriched via pre-amplification.*** *Delta Ct (BRAIN – UHRR) is highly correlated (R^2=0.969) with and without pre-amplification. 1µg RNA of each sample was processed with the Standard Protocol and 1ng of RNA was processed with the Pre-Amplification Protocol. The results show that 1µg of RNA processed with the Standard Protocol produced similar expression level differences as 1ng of RNA that was processed with the Pre-Amplification kit.*

coefficient of 0.969 (Figure 2). Figure 3 also depicts the individual results for two assays included in this gene panel.

Routinely used enrichment protocols have the tendency to increase the overall fluorescent background during analysis. Since the approach taken here is to selectively enrich transcripts of interest, it is not the result here (Figure 4).

WaferGen Biosystems adapted an easy multiplex pre-amplification protocol that will allow users to profile a large set of genes with initial RNA inputs as low as 10 picograms (pg).

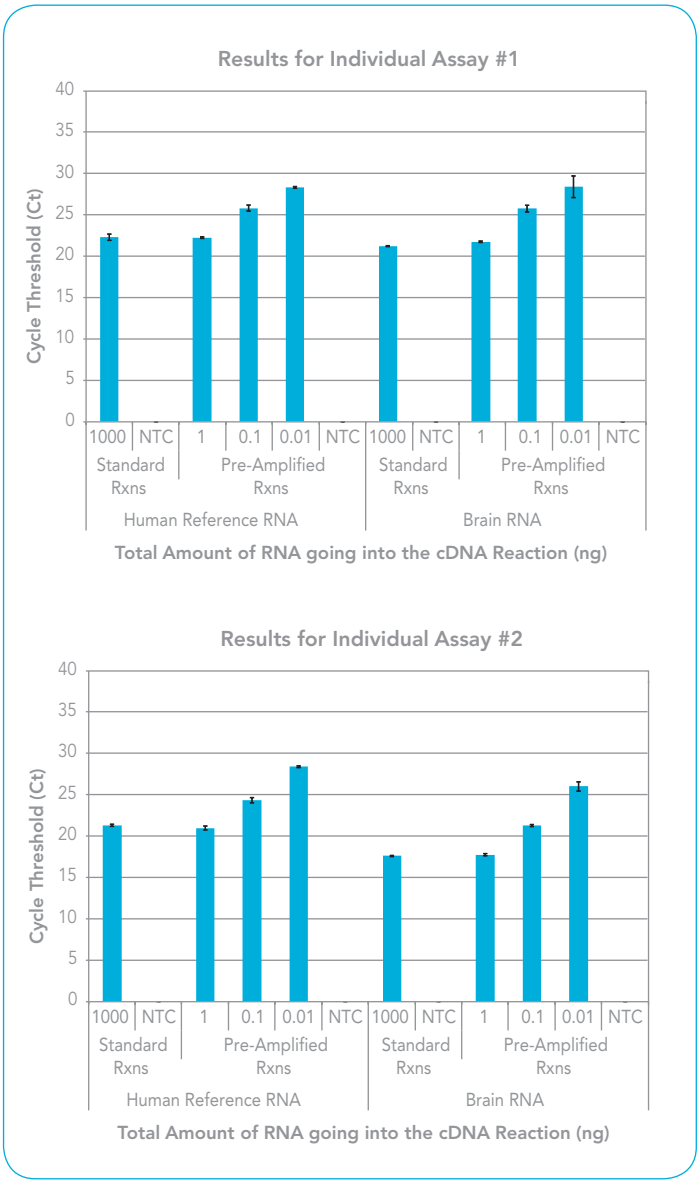


Figure 3. ***Standard cDNA reactions versus Pre-Amplified ones.*** *Samples containing Human Reference and Brain RNA were reverse transcribed and processed as described in the Materials and Methods section. These results demonstrate that pre-amplification of cDNA increased the detection of the 96 signatures included in this assay panel. Assay #1 and Assay #2 results are shown here. The finding showed that 1000ng of RNA processed with the Standard Protocol produced similar results as 1ng of pre-amplified RNA.*

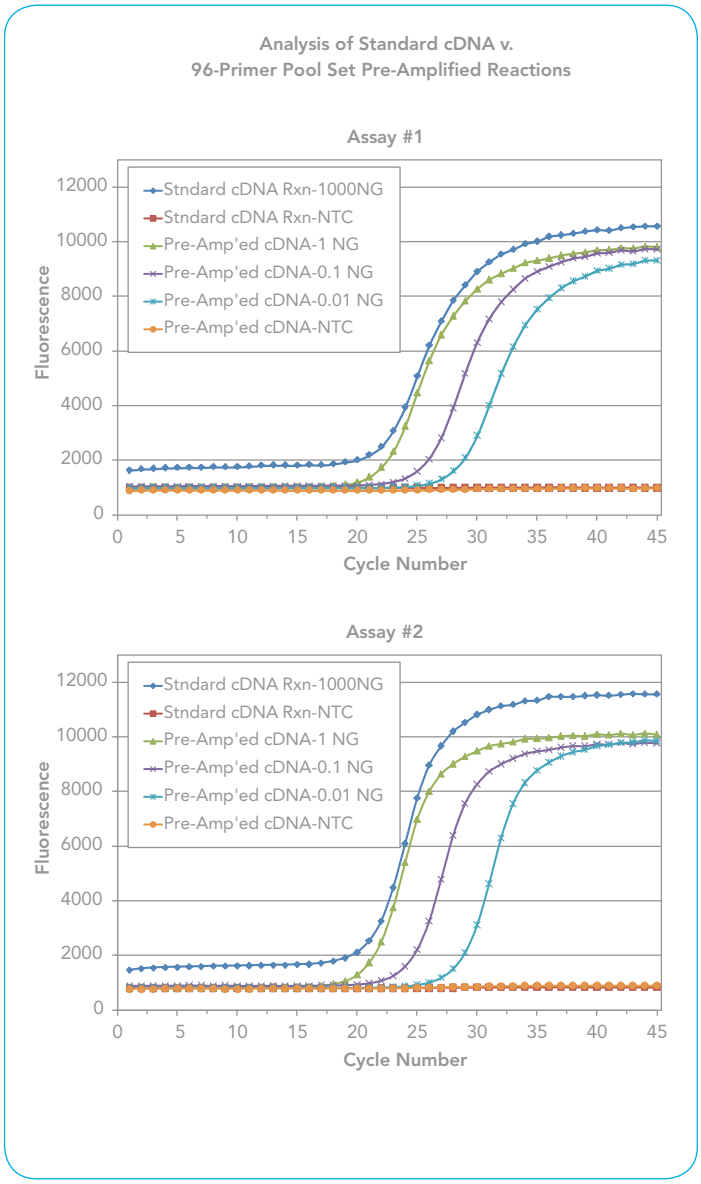


Figure 4. ***Fluorescence Background.*** *The enrichment of mRNA was targeted for specific transcripts, producing an increase in fluorescence background. Product from a cDNA library made with 1000ng of UHRR RNA input is also depicted as a comparison.*

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