Preamplification of Sample Limited Specimens for Real-Time Gene Expression Analysis (165)

Renata Coudry [1], Cynthia Spittle [2], and Junko Stevens [3], [1] Hospital A C Camargo, Rua Antonio Prudente 211, Liberdade, Sao Paulo. SP, Brazil.
[2] Fox Chase Cancer Center, 7701 Burholme Ave. Philadelphia, PA, U.S.A. [3] Applied Biosytems, 850 Lincoln Centre Drive, Foster City, CA, 94404

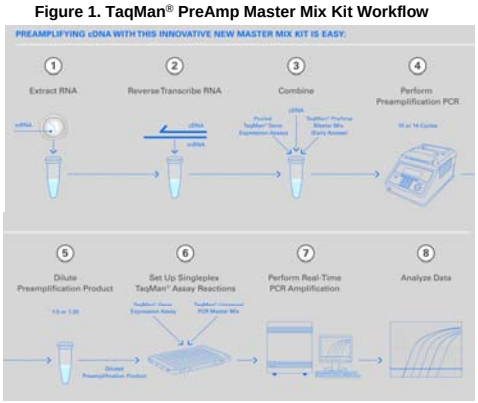
Abstract

Accurate gene expression profiling can be compromised by the quantity of RNA that is isolated from cells or tissues. We have developed a robust solution for uniform amplification of cDNA prior to quantitative, real-time PCR. TaqMan® Preamp Master Mix allows preamplification of up to 100 gene targets simultaneously using the TaqMan Gene Expression Assays as the source of pooled gene-specific primers. TaqMan assay-based preamplification preserves equilibrium of targets and retains the relative copy numbers of starting targets in a reproducible and precise manner. TaqMan® preamplification of random-primed cDNA is independent of amplicon distance from the 3' end, making it amenable to use with partially degraded or viral RNA.
Uniformity of preamplification was demonstrated using RNA extracted from cells obtained by Laser Capture Micro dissection (LCM). LCM was performed on formalin-fixed, paraffin embedded (FFPE) tissue sections. RNA was first reverse transcribed to cDNA using High-Capacity cDNA archive kit and preamplified using TaqMan Preamp Master Mix and pooled primers. Following preamplification, quantitative real-time PCR data was obtained for each target gene using universal cycling conditions on the ABI 7900 Sequence Detection System. This simple workflow enables researchers to enrich the amount of limited RNA samples uniformly and obtain gene expression profiles within 3 hours.

Introduction

The TaqMan® PreAmp Master Mix Kit provides users with a simple workflow that results in over a thousand-fold increase of cDNA in less than 1.5 hours. Figure 1 shows preamplification workflow. First, RNA is converted to single-stranded cDNA using AB High Capacity cDNA Archive Kit. Next, up to 100 TaqMan® Gene Expression Assays are pooled to create the assay pool. The assay pool is combined with the TaqMan® Preamp Master Mix and cDNA to create a reaction mixture that undergoes 10 or 14 cycles of PCR. The pre-amplified products are then diluted to eliminate any downstream assay inhibition and are used as the template for single-plex TaqMan® Gene Expression PCR.

Figure 1. TaqMan® PreAmp Master Mix Kit Workflow



The main objective when performing preamplification is to retain the gene expression profile of the original cDNA sample. We have developed the analysis method to assess uniformity of preamplification (ΔΔCt method). ΔΔCt method involves real-time relative quantitation PCR with cDNA as a starting template and preamplified amplicon as a starting template. For Human target, CDK1NB (HS00153277_m1) is used as a uniformity reference gene because of its consistent gene expression profile.

(1) For each target, preamplified ave. Ct are normalized to ave. Ct of uniformity reference gene

$$\Delta Ct_{preamp} = \text{ave. } Ct_{target\ X\ [preamp]} - \text{ave. } Ct_{uniformity\ reference\ gene\ [preamp]}$$

(2) Similarly, ave. Ct from cDNA template are normalized to ave. Ct of uniformity reference gene

$$\Delta Ct_{cDNA} = \text{ave. } Ct_{target\ X\ [cDNA]} - \text{ave. } Ct_{uniformity\ reference\ gene\ [cDNA]}$$

(3) ΔΔCt is determined by the difference of the two ΔCt

$$\Delta\Delta Ct = \Delta Ct_{preamp} - \Delta Ct_{cDNA}$$

A ΔΔCt close to zero indicates preamplification uniformity. Typically, 90% of TaqMan® Gene Expression Assays produce ΔΔ Ct within ± 1.5.

Pooling TaqMan® Gene Expression Assay Criteria

We recommend the following rules for pooling TaqMan® Gene Expression Assays.

(1) Pool TaqMan® assays with Ct< 35 when using 0.3 ng/μL cDNA.

(2) Do not include the 18S TaqMan® assay in the pool because it is highly expressed and introduces amplification bias.

(3) Check the uniformity of preamplification using ΔΔCt method.

Materials and Method

❑Degraded RNA Samples

Stratagene UHR Total RNA was degraded by adding 10 mM NaOH and heating at 37°C for 1 minute. The sample was immediately neutralized with 10 mM Tris, pH 7.0. The RNA integrity Number (RIN) was determined by Agilent 2100 Bioanalyzer. The degraded and untreated RNA samples were converted to cDNA using the AB High Capacity cDNA archiving kit. 25 ng of cDNA was used for 50 μL preamplification reactions.

❑Laser Capture Micro Dissection (LCM)

LCM was performed on FFPE mouse colonic sections using a Veritas™ System (Arcturus Bioscience, Mountain View, CA). Total RNA was isolated using the Paradise System (Arcturus Bioscience) and converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNA was then amplified using TaqMan® PreAmp Master Mix (Applied Biosytems).

❑Preamplification Reaction Conditions

Table 1. Preamplification Reaction Components

Component	Volume[a] (μL/Reaction)	Final Concentration
TaqMan PreAmp Master Mix (2X)	25.0	1X
Pooled assay mix (0.2X)	12.5	0.05X (each assay)
1–250 ng cDNA sample + nuclease-free water	12.5	0.02–50 ng/μL
Total	50.0	—

Table 2. Preamplification Thermal Cycling Conditions

	Enzyme Activation	Preamplification PCR	
	HOLD	CYCLE (10 or 14 cycles)	
		Denature	Anneal/ Extend
Temp	95 °C	95 °C	60 °C
Time	10 min	15 sec	4 min

Results

Figure 2. ΔΔCt values for the 1, 25 and 250ng cDNA

Preamplification reactions are plotted for the 96 test assays. The Δ Δ Ct values are along the Y-axis and the 96 test assays are plotted along the X-axis.

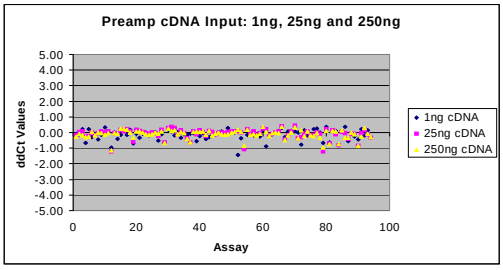


Figure 3. ΔΔCt from Degraded (RIN 7.4) and Untreated (RIN 9.0) RNA

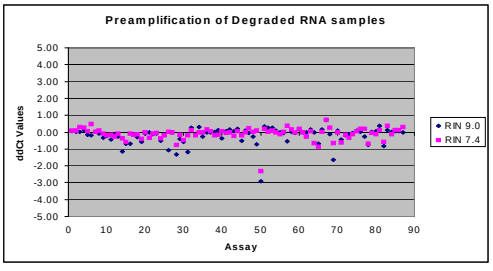
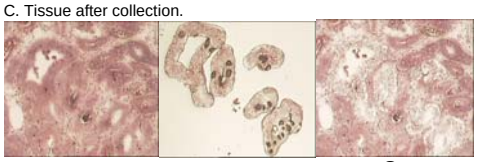


Figure 4. LCM and RNA Quality Evolution

A. Neoplastic colonic mouse tissue before LCM performance.
B. Neoplastic epithelial cells in the cap after being harvested.
C. Tissue after collection.



Bioanalyzer profiles of RNA extracted from 1,000 neoplastic colonic cells obtained from FFPE and frozen tissues using LCM. Electropherograms from frozen samples show distinct 18S and 28S peaks, demonstrating very good quality of the RNA. Although the 18S and 28S peaks are less distinct in the electropherograms from FFPE samples, these profiles are also consistent with good quality RNA.

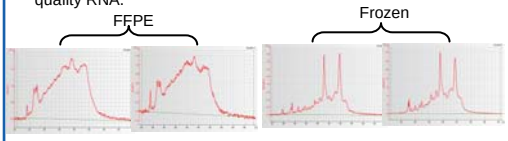


Figure 5. Preamplification Validation

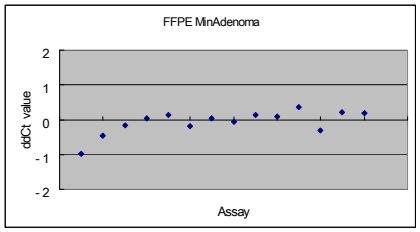
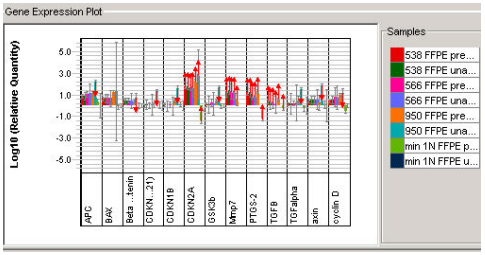
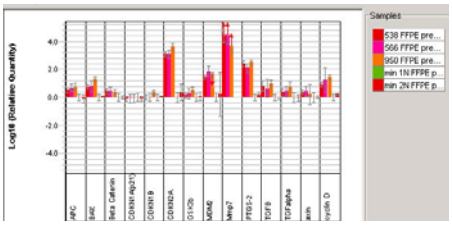


Figure 6. Gene Expression Profiling of Colonic Adenomas A: Unamplified cDNA, B: Preamplified cDNA

A 14-gene panel was used to compare the gene expression profile of normal colonic mucosa to adenomas in ApcMin/+ mice. Similar gene expression changes were observed in adenomas using both unamplified and pre-amplified cDNA samples. For example, we observed over- expression of the MMP-7 gene, an important target gene of the Wnt signaling pathway, in adenomas. These results demonstrate the use of TaqMan® Preamplification for gene expression profiling of limited samples.



A:Unamplified cDNA



B:Preamplified cDNA

Summary

TaqMan® PreAmp Master Mix and optimized cycling parameters enable nearly 100% efficient amplification of target sequences. Data obtained from the Applied Biosystems 7900HT Sequence Detection System demonstrate the wide utility of this process in many gene expression arenas including profiling of limited cell populations obtained from archived FFPE. Results using the TaqMan® PreAmp Master Mix Kit are amenable to partially degraded RNA samples.