LB-85

Gene Expression Profile of Microdissected Hepatocytes Infected with Hepatitis C Virus Using a Novel Multiplex PCR

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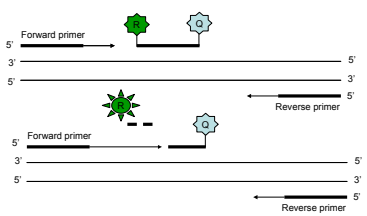
ABSTRACT

Accurate gene expression profiling can be compromised by the quantity and integrity of RNA that is isolated from cells or tissues. RNA amplification methods have been developed that produce sufficient quantities of RNA for the analysis of gene transcript levels using various platforms using as little as picograms of starting material. However, most of these methods are based on oligo-dT priming and are not suitable for use with fragmented or viral mRNA molecules. In order to overcome the limitations associated with current RNA amplification methods, we have tested the feasibility of using a combination of random and gene-specific primers to generate amplified cDNA for quantitative real-time PCR analysis. We have compared the relative levels of 17 different transcripts, including hepatitis C virus RNA, in samples containing 100-3000 human hepatocytes obtained by Laser Capture Microdissection (LCM). LCM was performed on frozen liver tissue sections using a Veritas System (Arcturus Bioscience, Mountain View, CA). Total RNA was isolated using the PicoPure RNA Isolation Kit (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 Biosystems). This kit allows up to 100 gene targets to be pre-amplified simultaneously using TaqMan® Gene Expression Assays as the source of pooled gene-specific primers. Quantitative real-time PCR data was obtained from the ABI PRISM® 7900 Sequence Detection System (Applied Biosystems). This protocol allowed nearly 100% efficient amplification of our target sequences and the results were reproducible using varying numbers of cells as starting material. Comparisons with unamplified material demonstrated that the relative copy number of our starting target sequences was maintained and that amplification was gene-specific. 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. This new amplification method will expand the use of gene expression profiling to characterize limited cell populations, including virus-infected cells.

Real-Time PCR Advantages for Gene Expression Analysis

Although microarray based platforms allow for simultaneous evaluation of thousands of genes in a single experiment, this technology can only estimate differences in gene expression levels. Real-time PCR is a more sensitive technique for detecting small changes in gene expression levels. Real-time PCR also permits gene expression analysis without the 3' bias commonly found in probes used in DNA microarray.

TaqMan® Assay



Fluorogenic 5' nuclease chemistry. Forward and reverse primers are extended with *Taq* polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the gene sequence between the two primers. As the polymerase extends the primer, the probe is displaced. An inherent nuclease activity in the polymerase cleaves the reporter dye from the probe. After release of the reporter dye from the quencher, a fluorescent signal is generated.

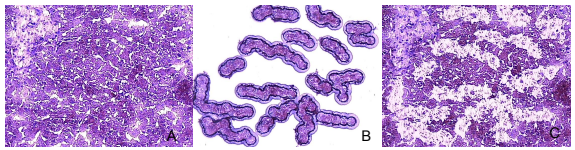
METHODOLOGY

⌘ LCM:
- Liver frozen samples (5µm sections and H&E staining)
- Veritas™ System (Arcturus Bioscience)
- 20 to 1,000 microdissected cells
- RNA Isolation: PicoPure RNA Kit (Arcturus Bioscience)
- RNA quality evaluation: Agilent 2100 Bioanalyzer

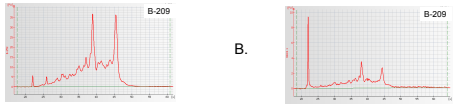
⌘ Preamplification (ABI PRISM 7900 Sequence Detection System)
- cDNA synthesis: High Capacity cDNA Archive Kit (Applied Biosystems)
- TaqMan® Assays: 17 assays
- TaqMan® PreAmp Master Mix
- 10-14 cycles

⌘ Real-Time PCR amplification and analysis (ABI PRISM 7900 SDS)
- Pre-amplified and un-amplified cDNA
- TaqMan® Gene Expression Master Mix
- TaqMan® Gene Expression Assay

LCM and RNA Quality Evaluation

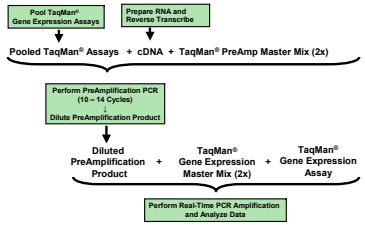


A. Liver human tissue before LCM performance. **B.** Hepatocytes in the cap after being harvested. **C.** Liver tissue after collection

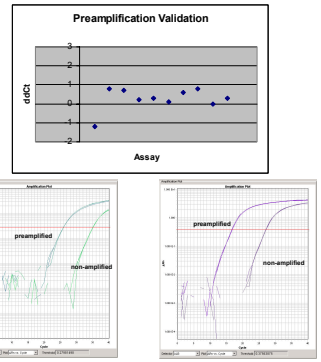


Bioanalyzer profile from 1,000 (A) and 20 (B) hepatocyctes collected using LCM. Both eletropherograms show 18 and 28s peaks, demonstrating very good quality of the RNA.

Steps from cDNA Synthesis to RT-PCR Analysis



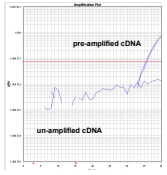
COMPARISON OF NON-AMPLIFIED TO PREAMPLIFIED cDNA



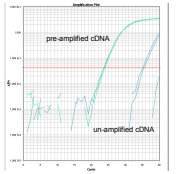
Beta actin Albumin

Total RNA isolated from hepatocytes was converted to cDNA using the High Capacity cDNA Archive Kit. Preamplification was performed for 10 cycles using the TaqMan® PreAmp Master Mix Kit with a 17-plex primer pool. 5 µl of diluted preamplified cDNA was used in each real-time reaction. An equal volume of non-amplified was used in parallel reactions. Efficient amplification (ddCt ≤ ±1.0) of 9/10 target sequences was observed.

RESULTS



Amplification plot for beta actin showing successful detection of beta actin cDNA in pre-amplified sample but no detection in un-amplified sample using 20 cells.



Amplification plot for HCV showing improved detection of viral cDNA levels in pre-amplified sample compared to un-amplified sample using 100 cells.

CONCLUSIONS

⌘ Each primer/probe set must be validated for efficient and linear preamplification by comparing the dCt values between unamplified and preamplified material
⌘ cDNA preamplification is not dependent on oligo-dT based priming and is therefore suitable for studying the modulation of specific mRNA levels caused by viral infection
⌘ It is possible to perform gene expression analysis with a minimum of 20 cells using the TaqMan® PreAmp Master Mix technology
⌘ Relative quantitation of low level transcripts is possible with TaqMan® PreAmp Master Mix

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