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Improved Application-Specific cDNA Array Analyses with AmpoLabeling-LPR: A New Probe Synthesis and Labeling Method for SuperArray GEArray™

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Abstract
The AmpoLabeling-LPR Kit protocol utilizes experimental RNA samples to synthesize labeled cDNA probes for hybridization to our gene-specific cDNA arrays, the GEArray™. The method improves gene expression profiling sensitivity and accuracy over conventional methods, and provides gene expression profile results similar to that of RT-PCR. The protocol is simple; it can be performed in a single microfuge tube in two hours.

Introduction
Synthesizing and labeling cDNA probes, like RNA preparation, is one of the critical factors in cDNA array analysis. The more abundant, specific and labeled the probe is, the more sensitive and accurate the array analysis will be. Conventional methods of probe synthesis and labeling that use only random hexamers or oligo-dT as primers are non-specific and result in a high background of probe binding and can yield false-positive or false-negative signals. Consequently, the observed relative gene expression profile becomes skewed. Our AmpoLabeling-LPR method, optimized for use with our focused and application-specific GEArray™ Original and Q and S Series gene arrays, avoids these difficulties. It detects low abundance messages ordinarily missed by conventional reverse transcription methods. RT-PCR results verify that the corresponding genes detected by AmpoLabeling-LPR are indeed expressed in the experimental RNA sample. Furthermore, the AmpoLabeling-LPR method, but not the conventional method, produces relative gene expression profiles that closely match RT-PCR derived profiles. The new probe labeling method is also ideal for applications where total RNA is limiting. As little as 0.1 micrograms of total RNA can be used. Larger amounts of RNA can also be accommodated, and the resulting signal intensity is proportional to the amount of input RNA. Multiple rounds of Linear Polymerase Replication (LPR) can be used, and the signal intensities are also proportional to the number of LPR cycles employed. Therefore, the use of AmpoLabeling-LPR derived probes increases the sensitivity of message detection in our GEArray™ systems, and through proper normalization, keeps your relative gene expression profiles unbiased.

General Procedure for Generating AmpoLabeling-LPR Probes:
A step-by-step protocol is included in the GEArray™ Kit User Manual and the Probe Labeling Kits Protocols manual. It involves three basic reactions:

Random Primer Annealing: RNA is combined with a mixture of non-specific primers, heated to 70 °C for 3 min, and cooled to 37 °C for 10 min.
Reverse Transcription Reaction: A pre-warmed cocktail of transcription buffer, RNase inhibitor, and reverse transcriptase are added. The mixture is incubated at 37 °C for 25 min. Heating at 85 °C for 5 min stops the reaction by inactivating the reverse transcriptase and hydrolyzing the RNA.
Linear Polymerase Replication: A cocktail of LPR Buffer, gene-specific primers, biotin-16-dUTP (or α-^{32}P-dCTP), and a thermostable DNA-dependent DNA polymerase is added to the completed RT Reaction. LPR cycles ensue using the following program: 85 °C, 5 min; 10 to 30 cycles of (85 °C, 1 min; 50 °C, 1 min; 72 °C, 1min); 72 °C, 5 min. The reaction is stopped immediately with EDTA.

The AmpoLabeling-LPR protocol (Figure 1) is therefore easy to use. It can be carried out in a single microfuge tube in two hours.

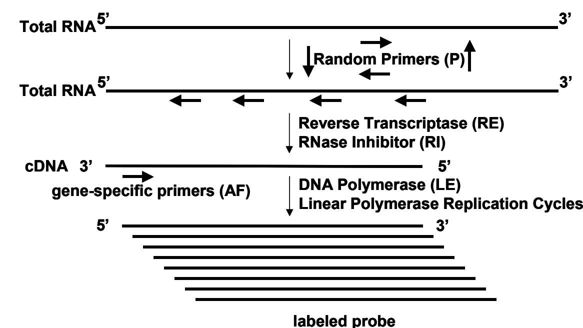

Figure 1: Diagram of AmpoLabeling-LPR Probe Synthesis and Labeling Protocol.

Proportionality With Input RNA and LPR Cycle Number

Probe amplification is the key to detecting low abundance messages and to using small amounts of RNA. Conventional methods of reverse transcription do not include an amplification step. As a result, they only detect medium or high abundance messages and require larger amounts of RNA. The results in Figure 2 demonstrate that overall array signal intensity increases with respect to increasing amount of input RNA used in the AmpoLabeling-LPR protocol. In fact, for several messages, the dependence is linear throughout the range tested. Furthermore, as little as 0.1 µg of total RNA can be used to achieve a gene expression profile. Figure 3 illustrates that overall array signal intensity also increases with respect to the number of cycles of linear polymerase replication (LPR) used. Some signals do saturate at high levels of input RNA (data not shown) or at a high number of cycles. However, with proper normalization, these phenomena do not affect relative gene expression profiling results.

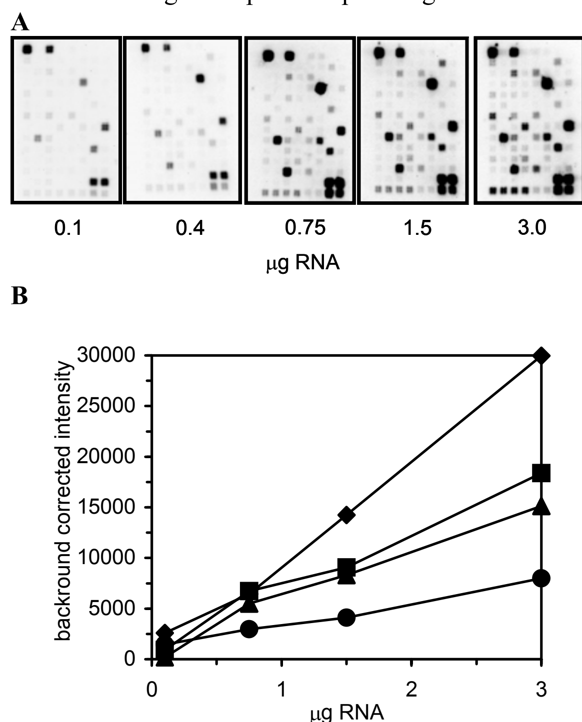

Figure 2: AmpoLabeling-LPR array signals are proportional to input total RNA. Different amounts (0.1, 0.4, 0.75, 1.5, 3.0 µg) of Human Universal Reference RNA (BD Biosciences) were used to synthesize biotin-labeled cDNA probe using 30 cycles of LPR. Each probe was hybridized to a separate membrane from the GEArray™ Q Series Human TGFβ/BMP Signaling Pathway Kit (HS-023N). Signals were detected using the Chemiluminescent Detection Method. Both procedures were performed according to the User Manual. Panel A displays the resulting arrays. Panel B plots the background corrected signal intensity versus input total RNA for a small sample of genes. (ID4, diamonds; BMPR2, squares; TCF8, triangles; TIMP1, circles.)

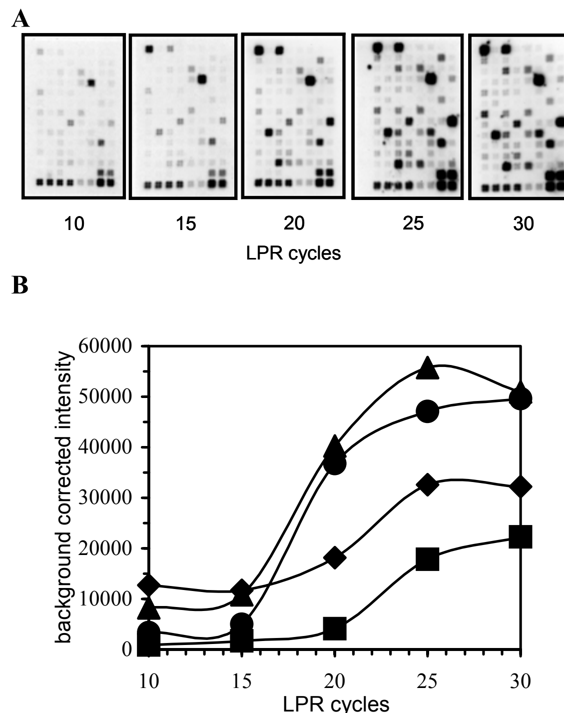

Figure 3: AmpoLabeling-LPR array signals are proportional to LPR cycle number. Human Universal Reference RNA (3 µg, BD Biosciences) was used to synthesize biotin-labeled cDNA probe in several separate probe synthesis reactions. The reactions were stopped after 10, 15, 20, 25, and 30 cycles of LPR. The probes were hybridized to separate membranes from the GEArray™ Q Series Human TGFβ/BMP Signaling Pathway Kit (HS-023N). Signals were detected using the Chemiluminescent Detection Method. Both procedures were performed according to the User Manual. Panel A displays the resulting arrays. Panel B plots the background corrected signal intensity versus cycle number for a small sample of genes. (MADH2, triangles; TGFBR1, circles; JUN, diamonds; BMPR1A, squares.)

Detecting Messages Ordinarily Missed by Conventional Methods

Figure 4 shows that the AmpoLabeling-LPR method detects more signals on our GEArray™ than the conventional method for probe labeling does. These unique signals could arise either because AmpoLabeling-LPR returns false positives or the conventional method returns false negatives. To test whether the AmpoLabeling-LPR results reflect genes that are actually expressed, the same input RNA was tested for the presence of messages corresponding to the unique spots using RT-PCR. In each case, these messages were detected by RT-PCR (Figure 4). Therefore, AmpoLabeling-LPR detects low abundance messages that conventional methods cannot.

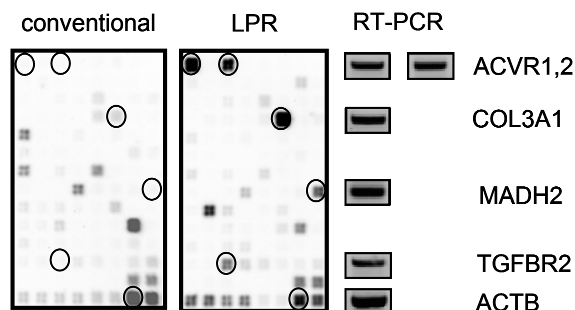

Figure 4: AmpoLabeling-LPR detects messages ordinarily missed by conventional methods but caught by RT-PCR. Human Universal Reference RNA (3 µg, BD Biosciences) was used to synthesize biotin-labeled probe using either MMLV reverse transcriptase or AmpoLabeling-LPR (30 cycles). The probes were hybridized to separate membranes from the GEArray™ Q Series Human TGFβ/BMP Signaling Pathway Kit (HS-023N). Signals were detected using the Chemiluminescent Detection Method. Both procedures were performed according to the User Manual. Displayed are the arrays resulting from the conventional method (left) and AmpoLabeling-LPR (middle). The circled messages were chosen for further RT-PCR analysis. The right panel displays the resulting RT-PCR products as characterized by agarose gel electrophoresis. Beta-actin is included as an internal control.

More Accurate Gene Expression Profiles

The goal of any gene array experiment is to compare the normalized expression level of a gene or a group of genes between two experimental conditions. To test the accuracy of gene expression profiles obtained by AmpoLabeling-LPR, the expression of insulin-related genes in mouse liver and thymus were compared using the conventional method, AmpoLabeling-LPR, and RT-PCR. Panel A of Figure 5 displays the qualitative gene expression profiles obtained by these three methods. The ratio of the corrected and normalized intensities from the liver and thymus experiments for each gene was calculated for each method. Panel B plots the ratios obtained from the conventional method versus those from RT-PCR for each gene. Panel C plots the ratios obtained from AmpoLabeling-LPR versus those from RT-PCR for each gene. The correlation factors for the two curve-fits show that the AmpoLabeling-LPR results correlate with RT-PCR results better than the conventional method results do.

Enhanced Gene Expression Profiling

The AmpoLabeling-LPR method for probe synthesis and labeling therefore provides more accurate and sensitive gene expression profiling results. It detects messages ordinarily missed by conventional methods. Its gene expression profiles resemble those obtained using RT-PCR. Also, a smaller amount of input RNA can be used to achieve gene expression profiles.

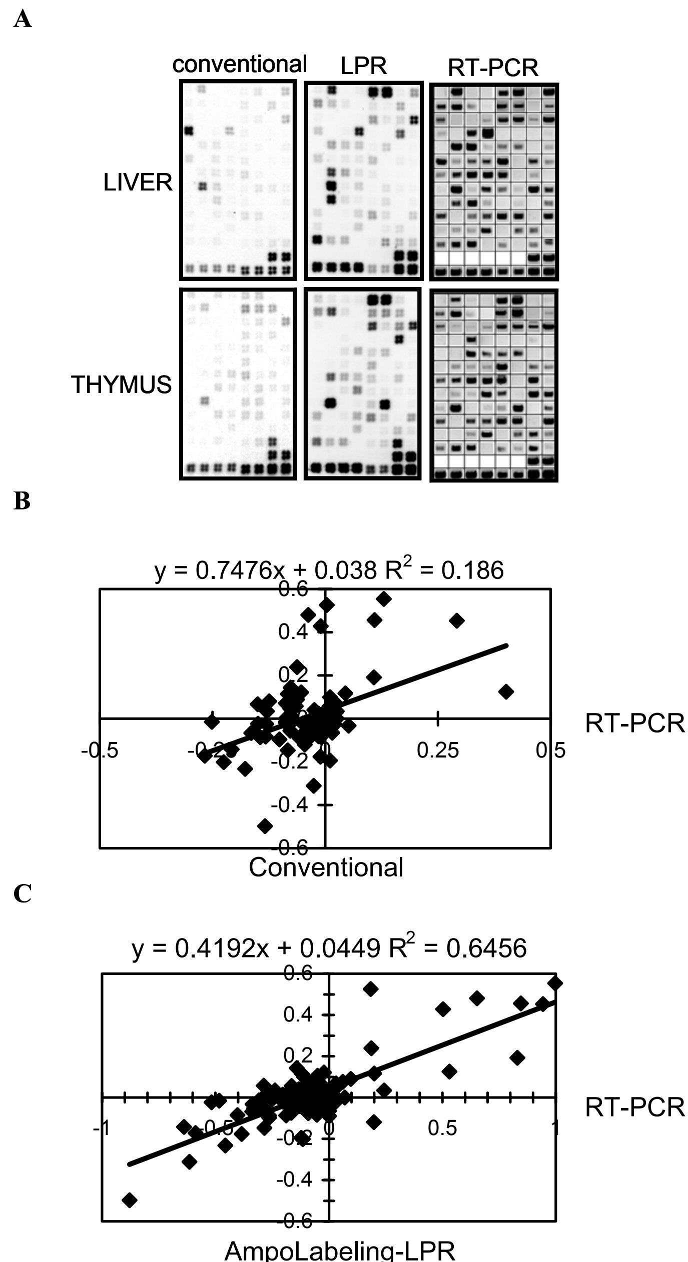

Figure 5: AmpoLabeling-LPR relative gene expression profile matches RT-PCR generated profile better than conventional method. Mouse liver or mouse thymus total RNA (7 and 5 µg, respectively, BD Biosciences) was used to synthesize biotin-labeled probe using either 30 cycles of LPR or MMLV reverse transcriptase. The probes were then used to hybridize separate array membranes from the GEArray™ Q Series Mouse Insulin Signaling Pathway Kit (MM-030N). Signals were then detected using the Chemiluminescent Method. All messages were also analyzed in a similar fashion using RT-PCR. Panel A displays the arrays from the GEArray™ analyses and a representation of the bands detected by agarose gel electrophoresis of the corresponding RT-PCR products. Panel B plots the ratio of liver to thymus expression of each gene as determined for RT-PCR (ordinate) and conventional labeling (abscissa). Panel C plots the ratio of liver to thymus expression of each gene as determined for RT-PCR (ordinate) and AmpoLabeling-LPR (abscissa). Both graphs are log-log plots.

The use of gene-specific primers also reduces the extent of endogenous RNA priming that can contribute to the appearance of false positive and high background signals. In this way, signal-to-noise ratios are greatly improved. Enhance your relative gene expression profile results with AmpoLabeling-LPR.

References:

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Sturzl, M., and Roth, W. K. (1990) *Anal. Biochem.* **185**, 164-169.
Millican, D.S., and Bird, I.M. (1997) *Anal. Biochem.* **249**, 114-117.

Protocols:

GEArray™ Q and S Series User Manual
(http://www.superarray.com/manuals/xpd_GEA_Q_Nonrad.pdf)

GEArray™ Probe Labeling KITS
(http://www.superarray.com/manuals/xpd_GEA_Label.pdf)

Ordering Information:

AmpoLabeling-LPR Kit:

Radioactive Detection	L-03
Chemiluminescent Detection	L-03N

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