

# Use of Formalin Fixed, Paraffin-Embedded RNA Samples in Amplification and Labeling Methods for Gene Expression Analysis

Several techniques are available for determining the integrity of an RNA sample such as denaturing agarose gel analysis<sup>1</sup>, the Agilent Bioanalyzer<sup>2</sup>, and the Arcturus Paradise QC process (Beta-Actin 3'/5' value)<sup>3</sup>. Most methods for RNA amplification and labeling rely on high quality, intact RNA samples. However, total RNA samples may become partially degraded due to inherent nucleases, handling, age, and method of preservation or extraction. In particular, formalin fixed, paraffin-embedded (FFPE) RNA samples are susceptible to degradation due to the preservation process. In order to test new strategies for the amplification and labeling of degraded RNA, we used chemically degraded RNA to mimic a FFPE sample. Then, we confirmed our methods by using FFPE RNA in our amplification and labeling kits to produce validated gene expression data.

**Problem:** Formalin fixed, paraffin-embedded samples, by nature, are partially degraded and contain low amounts of total RNA.

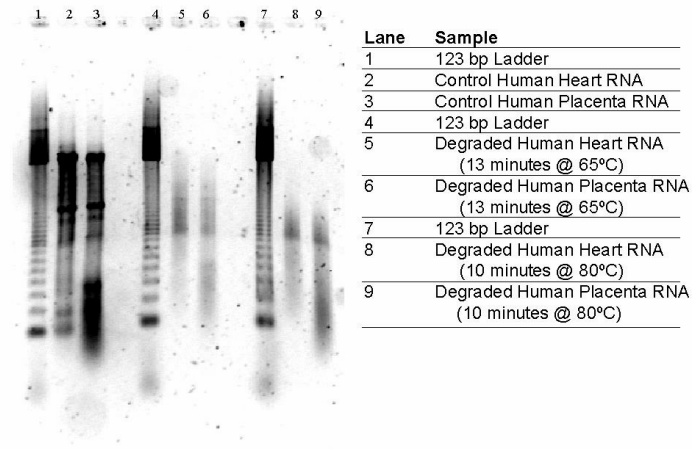
**Solution:** Genisphere's RNA amplification and labeling methods allow for the use of tiny amounts of these degraded total RNAs in gene expression analysis.

## Array Labeling

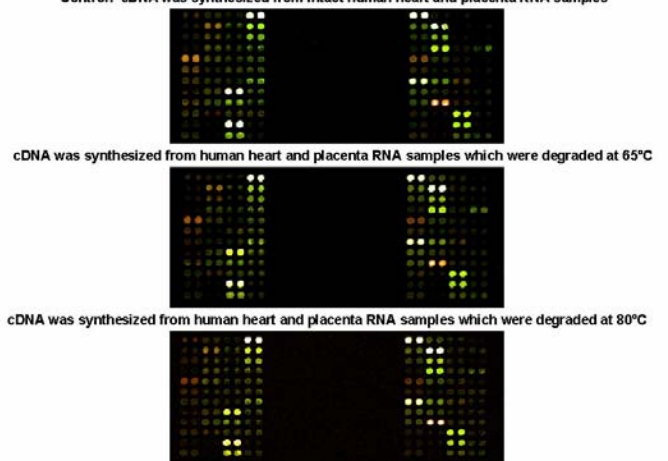
As previously reported by Genisphere, random prime reverse transcription, when combined with the 3DNA™ Dendrimer Labeling method, is a sensitive labeling technique for both intact and degraded RNA samples<sup>4</sup>. Figure 1 shows human cDNA arrays that were hybridized with random-primed cDNA from intact and degraded RNA samples.

**Figure 1.** Degraded sample analysis using Genisphere array kits. Differential gene expression analysis comparing intact RNA to partially degraded and almost completely degraded RNA samples, indicates that good quality array data can be generated from 0.5-2µg degraded RNA samples.

Magnesium Acetate Degradation of Total RNA Samples:  
Human heart and placenta total RNAs were degraded at various times and temperatures.



Use of Control (undegraded) and Degraded RNA samples in Genisphere's Random Prime Microarray Labeling Protocol  
Control: cDNA was synthesized from intact human heart and placenta RNA samples



### Gene Expression Profiling of Degraded RNA and Control (Intact) RNA on Microarrays:

- When comparing the heart/placenta differentials of control RNA to the heart/placenta differentials of RNA degraded at 65°C, a correlation coefficient of 0.90 was generated.
- When comparing the heart/placenta differentials of control RNA to the heart/placenta differentials of RNA degraded at 80°C, a correlation coefficient of 0.84 was generated.

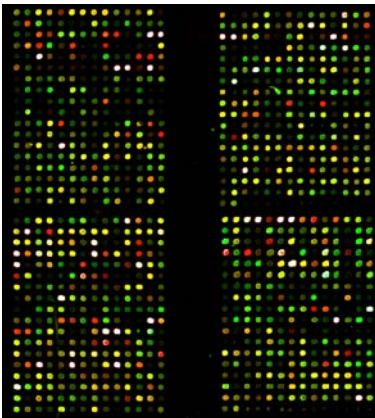
## RNA Amplification

Although 0.5-2 $\mu$ g degraded total RNA samples have been validated for array labeling, a linear RNA amplification method can still be needed for smaller sample sizes. An amplification procedure has been devised to enable use of random priming in the initial cDNA synthesis. The method, SenseAmp, produces amplified RNAs that are in the original, sense-strand orientation. Multiple rounds of amplification may be performed by repeating the process.

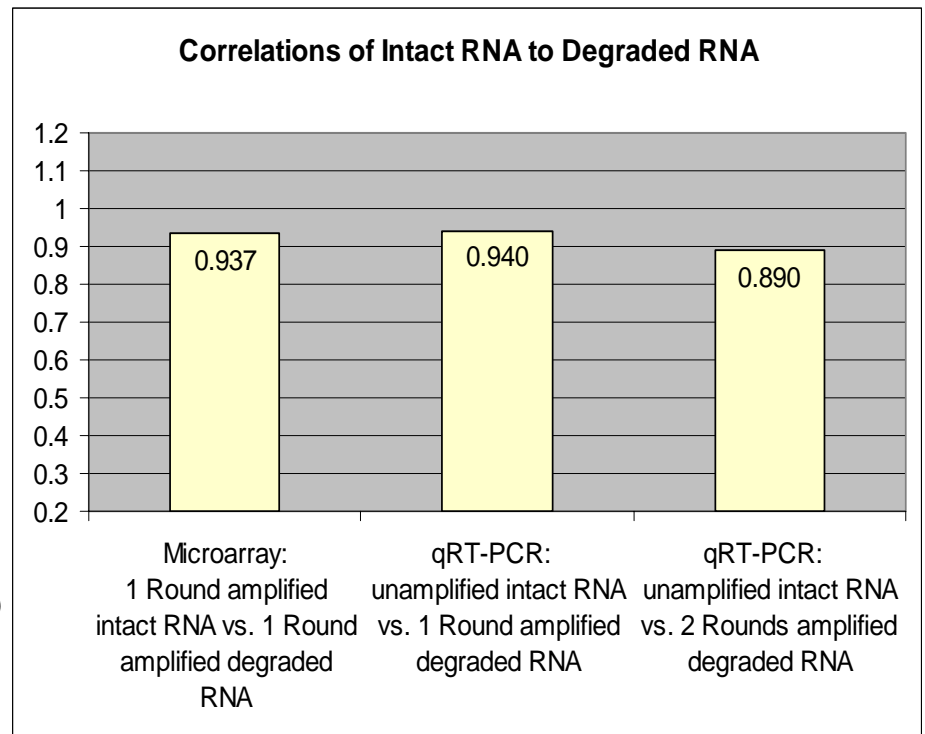
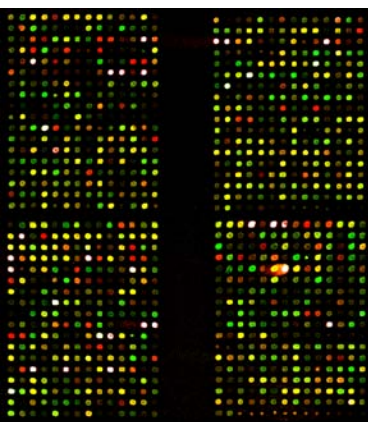
To test the amplification method with degraded RNA, total RNA samples from rat brain and rat liver were fragmented with magnesium acetate at 65°C for 10 minutes, to produce partially degraded RNA. 75ng aliquots of the degraded RNAs and 75ng aliquots of intact RNAs were amplified with the SenseAmp Plus kit. The amplified intact and degraded senseRNAs were labeled with the Array 900 kit and analyzed on 8K rat oligo microarrays to determine their expression profiles. To further validate the microarray data, the senseRNAs from the degraded samples (1 and 2 rounds of amplification) were compared to the original intact RNAs by qRT-PCR analysis of 192 genes (see Figure 2).

**Figure 2.** Microarray and qRT-PCR analysis comparing intact RNA to degraded RNA samples. Good quality gene expression data can be generated from 75ng degraded RNA samples that are amplified with SenseAmp Plus, using either one or two rounds of amplification.

Intact RNA, Amplified (1 Round)



Degraded RNA, Amplified (1 Round)



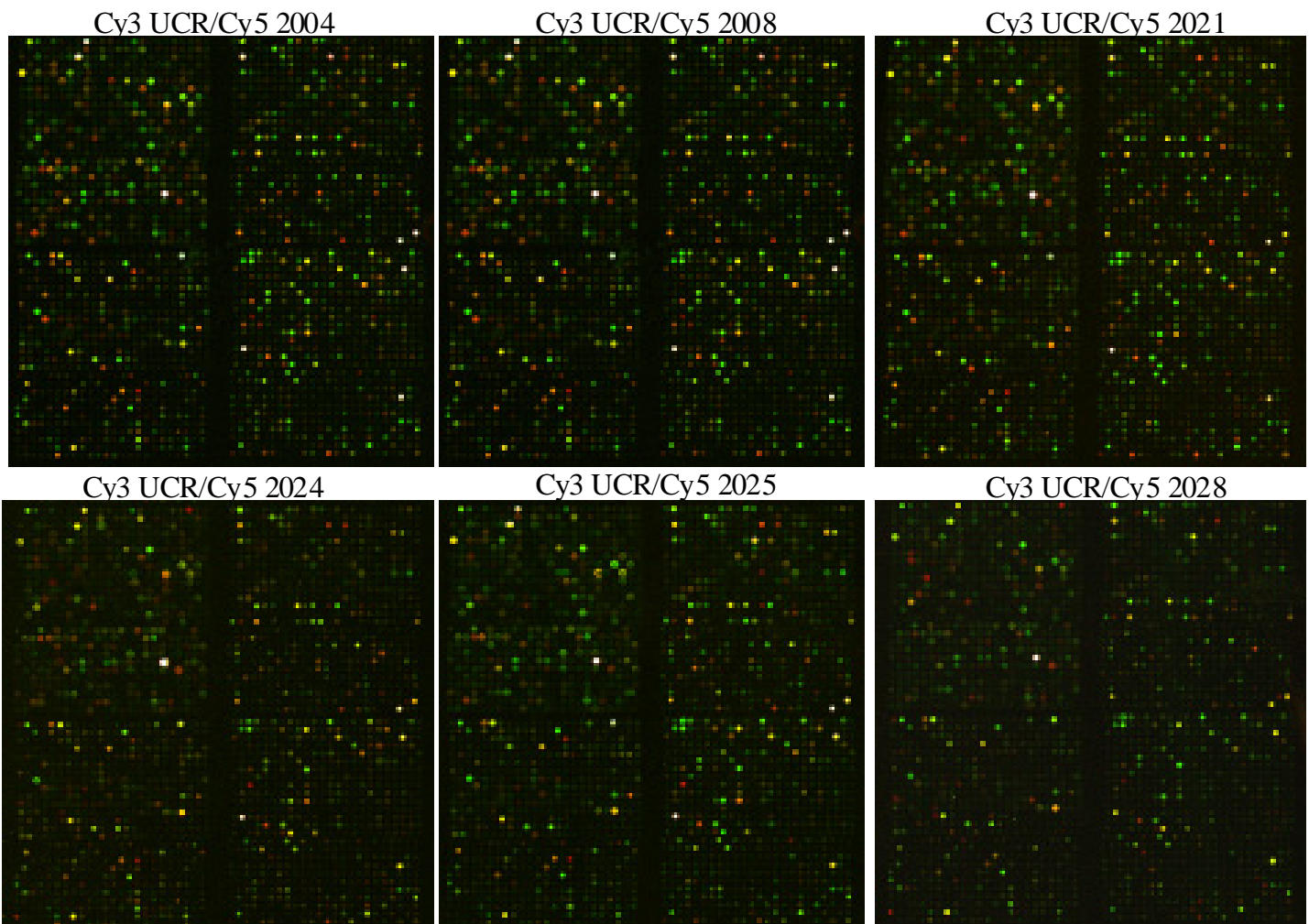
For microarray analysis, the brain/liver ratios of amplified intact RNA were compared to the brain/liver ratios of amplified degraded RNA. The ratios of approximately 4,000 genes were compared to generate the correlation of 0.937.

For qRT-PCR analysis, the brain/liver ratios of unamplified intact RNA were compared to the brain/liver ratios of amplified degraded RNA (either 1 or 2 rounds of amplification). The ratios of 192 genes were compared to generate the correlation of 0.940 (degraded RNA amplified in 1 round) or 0.89 (degraded RNA amplified in 2 rounds).

The performance of the SenseAmp Plus kit was further evaluated with FFPE RNA samples from Stanford University, that had been archived for 1-5 years. The FFPE samples had Arcturus Paradise QC metric ratings between 4 (intact) and 164 (severely degraded). Between 5 and 10 nanograms of the FFPE samples were amplified with SenseAmp Plus (2 rounds), labeled with the Array 900 kit, and hybridized to 19K human oligo arrays. Parallel amplification and labeling reactions were run with 10 nanograms of Universal Control RNA (Stratagene) (see Figure 3). Despite the broadness in degradation of the control and FFPE samples, the amplification process resulted in similar amounts of recovered senseRNA (~47µg), and the labeling kit generated consistent signals on the microarrays.

**Figure 3.** Quality gene expression data can be obtained from FFPE RNA samples using 2 rounds of SenseAmp Plus amplification and Array 900 labeling.

RNA Sample	Paradise QC Metric	SenseRNA Generated
Universal Control (UCR)	<10	50.6 µg
FFPE 2004	159	46 µg
FFPE 2008	4	44.3 µg
FFPE 2021	41	37.8 µg
FFPE 2024	22	51.1 µg
FFPE 2025	121	54.2 µg
FFPE 2028	164	46.8 µg



## Discussion and Conclusions

Small degraded RNA samples (0.5-2 $\mu$ g) can be labeled for microarray analysis and produce expression data that has a high correlation to intact samples. Reproducibility from array to array falls within the acceptable experimental range when labeling with the Genisphere random prime method, whether the samples are degraded or intact (data not shown). For successful use of smaller degraded RNA samples (5-10ng), the SenseAmp Plus RNA amplification kit is required, because it enables the use of a random primer in the initial step, first strand cDNA synthesis.

For this work, archived FFPE RNA samples were analyzed using the Arcturus QC method. The results indicated that the samples were not suitable for microarray experiments. However, Genisphere's RNA amplification and labeling methods allowed for the use of these samples in gene expression experiments. After two rounds of SenseAmp amplification, nearly equal amounts (~47 $\mu$ g) of senseRNA were recovered, regardless of the degree of degradation of the starting sample. Furthermore, these samples resulted in useful gene expression data from microarrays. Although we used Genisphere's Array 900 labeling kit for microarray hybridization, any standard method may be used to label senseRNA.

## Methods

**RNA Degradation:** All total RNAs (human heart and placenta, rat brain and liver) were purchased from Ambion. Aliquots were degraded by a magnesium acetate degradation process.

**SenseAmp Plus RNA Amplification:** RNA is primed using a random primer to produce single-stranded cDNA. This first strand cDNA is purified then tailed with dTTP using Terminal Deoxynucleotidyl Transferase. A special T7 Template Oligo is annealed to the 3' (tailed) end of the cDNA. Klenow enzyme fills in the 3' end of the cDNA to produce a double-stranded T7 promoter. (The T7 Template Oligo contains a blocker to prevent second strand synthesis.) Thousands of senseRNA copies are generated by T7 RNA polymerase in an overnight in vitro transcription reaction. Poly (A) tails are regenerated on all senseRNA molecules by Poly (A) Polymerase and ATP.

**Array Labeling and Hybridizations:** The following Genisphere labeling kits were used: Array 350RP (Figure 1), Array 900 (Figures 2 and 3). All hybridization conditions were performed according to the protocol of the labeling kit. Microarrays were provided by the University of Michigan (human cDNAs), the Keck Center at Rutgers University (rat 8K oligo), and the University of Arkansas (human 19K oligo).

**qRT-PCR:** Methods are published<sup>5</sup>.

## References

1. Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning, A Laboratory Manual (Second Edition) Cold Spring Harbor Laboratory Press, 1989.
2. Agilent 2100 Bioanalyzer analysis: <http://www.chem.agilent.com/Scripts/PDS.asp?lPage=51>
3. Arcturus Paradise™ Reagent System Quality Assessment Kit: [http://www.arctur.com/research\\_portal/products/paradise\\_main.htm](http://www.arctur.com/research_portal/products/paradise_main.htm)
4. Schwalm et al., Development of a Random Prime Labeling Method Using Fluorescent 3DNA Dendrimers and Use with Small Degraded RNA Samples, poster 60, Chips to Hits 2002.
5. Goff et al., Evaluation of sense-strand mRNA amplification by comparative quantitative PCR, BMC Genomics 5:76 (2004).