Essential Factors for a Successful Oligo GEArray® Experiment: Input RNA and cRNA Amounts

Introduction

Total RNA availability is usually the limiting factor in designing microarray experiments. Some sources of biological material, particularly small pieces of tissue, only yield small quantities of total RNA. Other projects may require the characterization of the same RNA sample across multiple microarrays for technical replicates or even different gene lists or pathways. Probe and RNA amplification methods involving reverse transcription followed by *in vitro* transcription (RT-IVT) are commonly used to label samples for microarray-based experiments. These well-characterized protocols amplify small amounts of RNA in an unbiased manner to produce sufficient labeled cRNA for microarray experiments.

The TrueLabeling-AMP™ 2.0 Kit is a simplified version of more traditional RT-IVT protocols designed and optimized for the Oligo GEArray® microarrays. The current recommendations call for the use of anywhere from 100 ng to 3 µg of total RNA in the protocol. But, how much RNA should actually be used in an optimal protocol? And furthermore, how reliable are the results when only small amounts of RNA are available? This technical note compares Oligo GEArray® analyses generated from different amounts of the same total RNA sample. The results demonstrate that gene expression profiles are not greatly affected by the amount of input material used.

Biotinylated cRNA Yield

We first compared the yield of biotin-labeled cRNA from different amounts of a universal source of total human RNA using the TrueLabeling-AMP™ 2.0 Kit protocol (Table 1). We find that larger amounts of input RNA and longer IVT reactions increase labeled cRNA yield. To meet the 2 µg cRNA requirement for the Oligo GEArray® hybridization protocol, at least 100 ng of total RNA and an IVT reaction time of at least 8 hours is needed.

Oligo GEArray® Hybridization and Detection

We next compared the microarray hybridization results using labeled cRNA generated from different amounts of input total RNA. The same amount of labeled cRNA (2 µg, when available) from each of the overnight (16-hour) reactions was hybridized to identical copies of an Oligo GEArray® pathway-focused microarray. The raw microarray images are displayed in Figure 1.

Table 1: The cRNA yield from the TrueLabeling-AMP™ 2.0 Kit protocol increases with increasing amounts of input RNA and with increasing IVT incubation time. Different amounts (3, 1, 0.5, 0.1, and 0.05 µg) of Human XpressRef™ Universal Total RNA (GA-004) were used to generate biotin-labeled cRNA using the TrueLabeling-AMP™ 2.0 Kit (GA-030). The *in vitro* transcription reactions were carried out for 4, 8 or 16 hours. After purification with the ArrayGrade™ cRNA Cleanup Kit (GA-012), cRNA yield was determined with a NanoDrop® spectrophotometer.

Input RNA (µg)	cRNA Yield (µg)		
	4 h IVT	8 h IVT	16 h IVT
3.0	11.00	17.80	23.60
1.0	6.90	11.25	15.80
0.5	3.90	7.50	10.75
0.1	1.20	2.40	3.55
0.05	0.80	1.49	1.81

The overall appearances of the images do not vary greatly between the different amounts of input total RNA used. The most notable exception is the microarray hybridized with less than two micrograms of labeled cRNA due to the lack of yield from a substandard amount of input total RNA. The intensity values summarized in Table 2 corroborate the consistency of the microarray results.

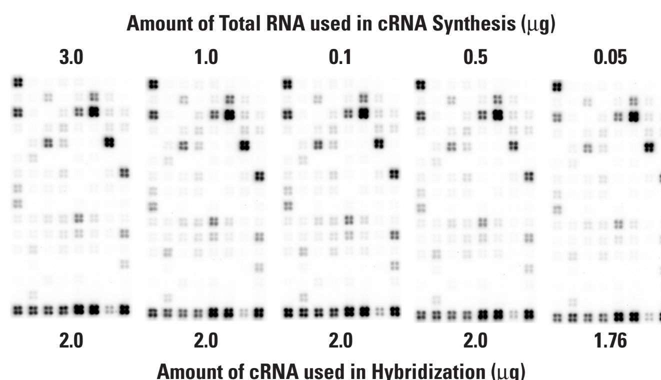

Figure 1: Biotinylated cRNA synthesized from different amounts of total RNA starting material yield similar microarray images. When available, a constant amount (2 µg) of labeled cRNA generated from the different amounts of total RNA described in Table 1 were hybridized to different copies of the Oligo GEArray Human Tumor Metastasis Microarray HybTube Format (OHS-028) for 18 h at 60°C. Note that only 1.76 µg cRNA from the smallest amount of total RNA could be used for hybridization. After the washing and chemiluminescent detection steps described in the User Manual, the images were acquired with an Alpha-Innotech FluorChem station with 20 minute exposures.

Table 2: Using different amounts of total RNA does not drastically affect overall microarray signal intensity. Raw signal intensities for individual genes from the microarray images in Figure 1 were extracted and analyzed using the GEArray® Expression Analysis Suite software, and were then summarized in the table. Genes are considered "Absent" if their average signal intensity is less than the mean value of the local backgrounds of the lower 75 percentile of all non-bleeding spots. All other genes are considered "Present".

	Amount of Total RNA used in cRNA Synthesis (µg)				
	0.05	0.1	0.5	1.0	3.0
Total Intensity	455466	570895	708709	658525	598921
Maximum Intensity	63907	63888	63882	63898	63353
Minimum Intensity	130	164	207	189	163
Mean Intensity	4031	5052	6272	5828	5300
Median Intensity	814	1276	1586	1551	1285
Present Calls	73	74	75	76	75
Absent Calls	40	39	38	37	38
Percent Present Calls	64.6	65.5	66.4	67.3	66.4

The total, maximum, mean, median, and minimum signal intensities are very similar when the recommended amounts of input total RNA are used. These values drop only slightly when less than the recommended amount of both input total RNA (50 ng) and less labeled cRNA are used instead. There is also no significant loss in the present call (the number of genes actually detected by the microarray experiment) across or even slightly below the recommended range.

We also compared the normalized signal intensity for each gene on each microarray in turn with the corresponding signal observed on the array hybridized with cRNA from the 3-µg labeling reaction (Figure 2). The consistent strong correlation to a straight line with a slope of one in each case indicates that the same level of expression can be observed from each amount of input RNA, as long as the same amount of cRNA is added to the hybridization. There is no significant overall decrease of signal intensity with different total RNA inputs.

The slight decrease in signal intensity seen at the 50 ng input level apparently comes in most part from the reduced amount of cRNA used (1.76 instead of 2 µg) in the hybridization step rather the actual amount of total RNA used for cRNA labeling. We have previously seen that array signal intensity is directly related to the amount of cRNA used during hybridization.

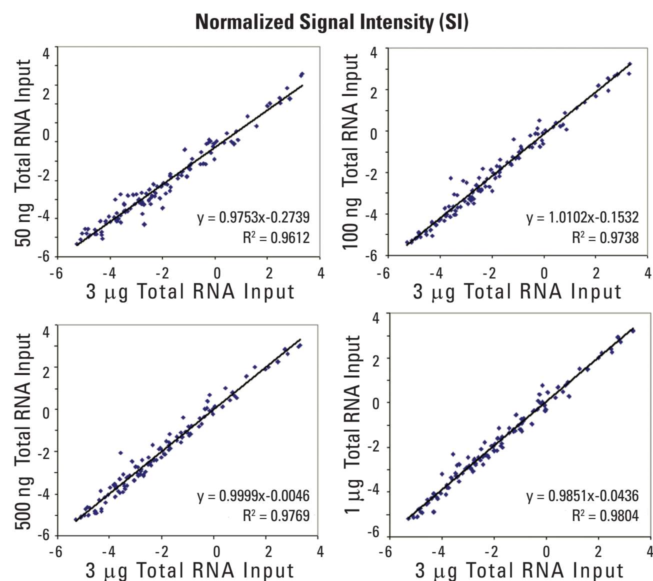

Figure 2: Different amounts of total RNA do not drastically affect the expression profile of individual genes. The normalized expression level of individual genes obtained as described in Table 2 using 3 µg of total RNA was compared to the corresponding data using other amounts of total RNA described in Table 1 and Figure 1. The normalization factor for each microarray was calculated as the average signal intensity of all the genes whose signals are present and unsaturated, excluding blank and control genes. Each of the datasets can be fit to a straight line with a slope very close to one (1) and a very high correlation factor ($R^2 > 0.97$).

Conclusions

The results indicate that the entire range of input total RNA recommended by the TrueLabeling-AMP™ 2.0 Kit procedure (0.1 to 3.0 µg) yields satisfactory results from the Oligo GEArray®. Furthermore, no differences in gene expression profiles, and thus no amplification biases, are observed whether using 3 µg or even as little as 50 ng of total RNA. However, to insure consistent results, we highly recommend always using the same amount of both input total RNA and labeled cRNA for all samples.

To insure the maximum yield from smaller amounts of total RNA input material (< 0.5 µg), longer IVT reactions (up to 16 hours) are needed. When sample RNA is very limited, no more than 2 µg cRNA can be generated even after an overnight IVT reaction meaning that samples can only be characterized on single microarrays. Shorter (1 to 4 hour) incubations are sufficient when analyzing larger amounts of input RNA (> 0.5 µg) on individual microarrays. However, longer IVT reaction times produce a greater quantity of labeled cRNA useful for multiple microarray hybridizations of the same sample.