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Technical Note

The New GeneChip® IVT Labeling Kit: Optimized Protocol for Improved Results

This technical note describes performance characteristics of the new Affymetrix GeneChip® Expression 3′-Amplification Reagents for IVT Labeling (IVT Labeling Kit). The kit is based on a novel biotinylated ribonucleotide analog (pseudouridine) that is incorporated in the T7 polymerase-mediated *in vitro* transcription (IVT) reaction to label and amplify cRNA targets for GeneChip brand arrays. This IVT Labeling Kit was developed as part of the new generation GeneChip expression 11-µm feature size array platform (2.0 Platform), with optimized hybridization and washing conditions, to provide consistent quality and performance. It replaces a previously recommended Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Kit) in both the One-Cycle and Two-Cycle Target Labeling Assays.

Results indicate that, by using the new IVT Labeling Kit, much of the detection and comparison data previously generated with the Enzo Kit can be confirmed and verified, when using either the 11-µm or 18-µm arrays. Platform performance improvements were observed, and these enhancements were found to be more prominent when used with the high-density, 11-µm arrays particularly with respect to increased discrimination. This led to increased sensitivity, as well as reduced false positives or improved specificity.

Two additional benefits of the IVT Labeling Kit are a new, streamlined overnight incubation protocol and a reduction in the minimum starting material requirement for the One-Cycle Target Labeling Assay to 1 µg of total RNA. Such reduction in starting material expands the range of samples used, providing greater flexibility for users.

Introduction

The GeneChip® Expression 3'-Amplification Reagents for IVT Labeling (IVT Labeling Kit) utilizes a single-label formulation based on a biotinylated pseudouridine molecule. This biotinylated label is combined with unlabeled ribonucleotides to make up the IVT Labeling NTP Mix. The "nucleotide to label" ratio and concentration of the MEGAscript® Reagent in the IVT Labeling Enzyme Mix (manufactured by Ambion for Affymetrix) were optimized to achieve a balance of robust cRNA yield, biotin incorporation, and array results, from as low as 1 µg of total RNA in the standard One-Cycle Target Labeling Assay.

A slightly different hybridization buffer, compared with previous recommendations, was found to work optimally with targets prepared with the new IVT Labeling Kit. The inclusion of 10% DMSO in the hybridization cocktail improved the discrimination between the Perfect Match (PM) probes and the Mismatch (MM) probes, therefore increasing the overall assay sensitivity. For more detailed information, please see the technical note: *GeneChip® Expression Platform: Comparison, Evaluation, and Performance*, available at www.affymetrix.com.

In addition, washing and staining steps on the new 49- and 64-format, 11-µm feature size arrays, such as GeneChip® Human Genome U133 Plus 2.0 (HG-U133 Plus 2.0) and GeneChip® Mouse Genome 430 2.0 Arrays, were optimized for targets prepared using the new IVT Labeling Kit, and now use higher stringency. A new fluidics script was developed based on these conditions and may be downloaded from www.affymetrix.com.

The existing fluidics scripts used for the 100-format, 11-µm feature size arrays, such as HG-U133A 2.0 and Mouse Genome 430A 2.0 Arrays, already utilize the more stringent conditions and, therefore, remain unchanged when targets prepared with the new IVT Labeling Kit are hybridized to these arrays.

A series of experiments was conducted to compare the array data obtained using the two labeling kits. The results are summarized below:

- Basic Array Metrics: common quality metrics were used to compare the array results obtained using the two different labeling reagents. In many tissues, the new IVT Labeling Kit generated slightly higher percent Present calls and reduced average signal intensity on the 11-µm arrays. The average background, noise, and 3'/5' ratios of housekeeping genes were highly similar.
- Detection Call Sensitivity and Specificity: with the new IVT Labeling Kit, spike-in transcripts on ROC curves were detected with 96 percent and 99 percent sensitivity at approximately 1:200,000 and 1:100,000 concentrations, respectively, with a specificity of 95.5 percent. The probability of any single transcript at the above concentrations to be detected in these experiments was equal to or better than the probability of detecting the same transcript with the Enzo® BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Kit).

- Change Call Sensitivity and Specificity: samples were prepared with transcripts spiked in at mRNA complexity ratios of 1:50,000, 1:100,000, 1:200,000, and 1:400,000. The data generated using the new IVT Labeling Kit showed detection of two-fold changes between the 1:200,000 and the 1:100,000 samples, and between 1:400,000 and 1:200,000 samples at a sensitivity of 88 percent and 64 percent, respectively. Both cases had a specificity of 99.5 percent, exceeding the performance of data obtained using the previously recommended Enzo Kit.
- Probe Set Signal Analysis: a tissue panel was used to analyze the tissue-specific responsiveness at the probe set level. Two-Way ANOVA indicated that the majority of the responsiveness observed using the previously recommended Enzo Kit was confirmed and reproduced with the new IVT Labeling Kit. However, differences were found to exist in terms of signal intensity for some probe sets.

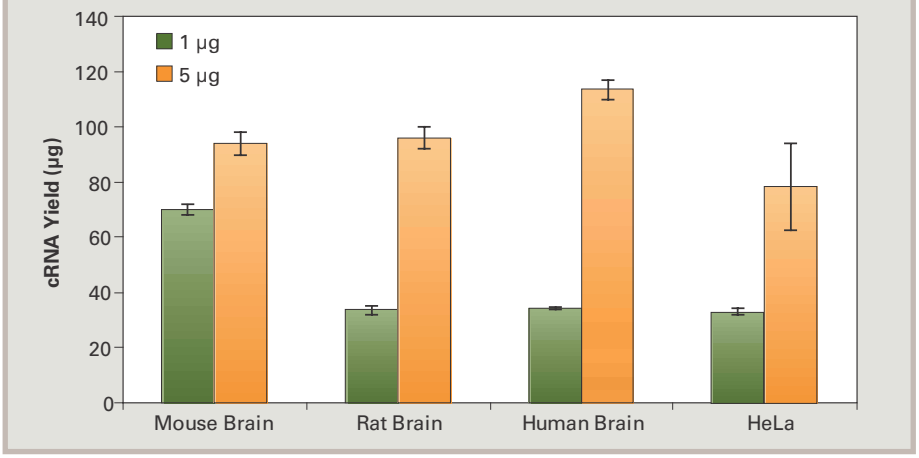
Results indicate that the new IVT Labeling Kit is compatible with all GeneChip expression reagents, including the Poly-A RNA Controls, One- and Two-Cycle cDNA Synthesis Kits, the Sample Cleanup Module, and Hybridization Controls. Furthermore, when these reagents are used together, optimal results are obtained from GeneChip brand arrays in terms of sensitivity, specificity, and reproducibility.

Results

cRNA YIELD AND LENGTH

The cRNA yield was evaluated in several tissues. Representative results are shown in Figure 1. Following the recommended protocol for 16-hour IVT incubation, between 80 and 110 µg of cRNA were obtained with 5 µg of total RNA as starting material in all four tissues. With 1 µg of total RNA from the same samples, cRNA yields of 30 and 70 µg were observed—sufficient for hybridizing to at least two GeneChip arrays in parallel.

Figure 1. cRNA yields using the IVT Labeling Kit. Starting with 1 µg or 5 µg of total RNA, cRNA was prepared using the recommended protocol with three technical replicates of each tissue. Average yields and standard deviations are shown.



The length of cRNA products generated using the new IVT Labeling Kit and the previously recommended Enzo Kit were compared on a Bioanalyzer. As seen in Figure 2, the new IVT Labeling Kit produced slightly shorter unfragmented products. After fragmentation, both cRNA targets were the same length.

Figure 2. Unfragmented and fragmented cRNA target length. The size of cRNA targets was measured on an Agilent 2100 Bioanalyzer. For unfragmented samples, the IVT Labeling Kit (brown line) produced slightly shorter transcripts than the previously recommended Enzo Kit (blue line), with approximately 10 to 15 percent average difference between the two methods. When fragmented, both methods produced average fragments of similar size, with the peak at roughly 100 bases. Standard size markers were used and the peaks in green represent 200, 500, 1,000, 2,000, 4,000, and 6,000 bases from left to right, respectively.

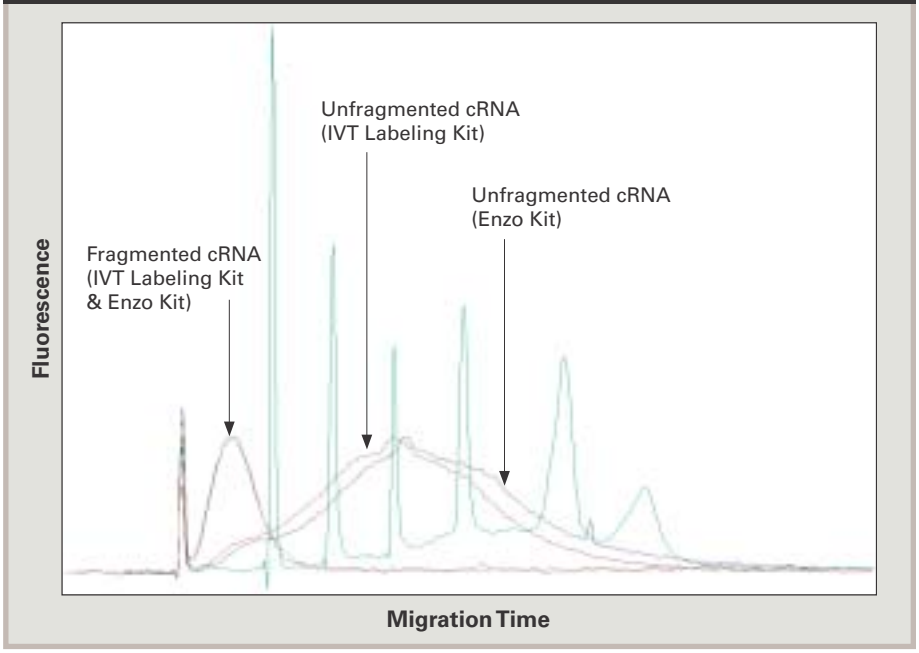
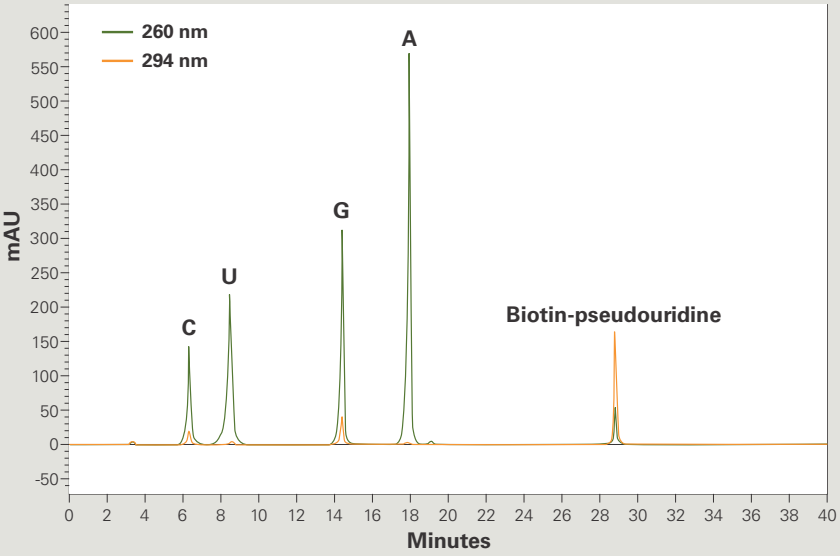


Figure 3. HPLC trace for biotin incorporation analysis. Labeled cRNA target was digested into individual nucleosides and then separated by HPLC and detected as distinct peaks. The four naturally occurring nucleosides, C, U, G, and A, were monitored by absorbance at 260 nm, shown in green. Absorbance of the biotinylated pseudouridine molecule was measured at 294 nm, shown in orange. Area-under-the-peak measurements were used together with the characteristic extinction coefficient of each nucleoside to calculate relative proportions of each component. The ratio between cold nucleosides and the biotin pseudonucleoside was calculated to determine incorporation efficiency. mAU = milliabsorbance units.



BIOTIN INCORPORATION

Analytical techniques were developed by Affymetrix to monitor the amount of biotin incorporated into cRNA targets. Such tools were used during development to optimize the final formulation of the kit. Furthermore, they will be used routinely during kit manufacturing to assist in obtaining a quantitative assessment of the kit's labeling efficiency, thereby ensuring consistency in reagent manufacturing from lot to lot.

Briefly, purified cRNA targets were treated with nuclease to create mononucleotides. The phosphate group was then removed with phosphatase to generate individual nucleosides. The samples were subsequently run on HPLC to resolve the different component peaks that were quantified to determine the biotin incorporation efficiency. A typical HPLC profile is shown in Figure 3.

The amount of biotin incorporated into cRNA may differ depending on the tissue type. As seen in Figure 4, the biotin incorporation was plotted as the number of biotins per 100 bases, which varied over the four different tissues. The single label reagent used in the new IVT Labeling Kit provided slightly lower biotin incorporation efficiency compared to the previously recommended dual-label Enzo Kit. However, based on array data described below, the biotin incorporation rate of the new IVT Labeling Kit is sufficient for equal or better sensitivity compared to the previously recommended Enzo Kit.

BASIC ARRAY QUALITY METRICS

Performance on GeneChip arrays using the new IVT Labeling Kit was compared to conventional labeling methods, with respect to basic array quality metrics. To ensure high-quality results across a wide range of samples, a large number of tissue and cell line samples was tested during development of the new kit. Sample summary results are shown in Table 1.

Targets were prepared from a human skeletal muscle total RNA sample in triplicate, using either the new IVT Labeling Kit or the Enzo Kit and hybridized to HG-U133A and HG-U133 Plus 2.0 Arrays. Two separate lots of the new IVT Labeling Kits (IVT 1 & IVT 2) were used to demonstrate lot-to-lot reproducibility.

Figure 4. Biotin incorporation rates comparing the new IVT Labeling Kit and previously recommended Enzo Kit across various human tissues. Using the analytical HPLC approach described earlier, the biotin incorporation efficiency was obtained from targets prepared from four different tissues, using two different labeling kits. The y axis represents the number of biotins incorporated per 100 bases (the size of an average cRNA target after fragmentation).

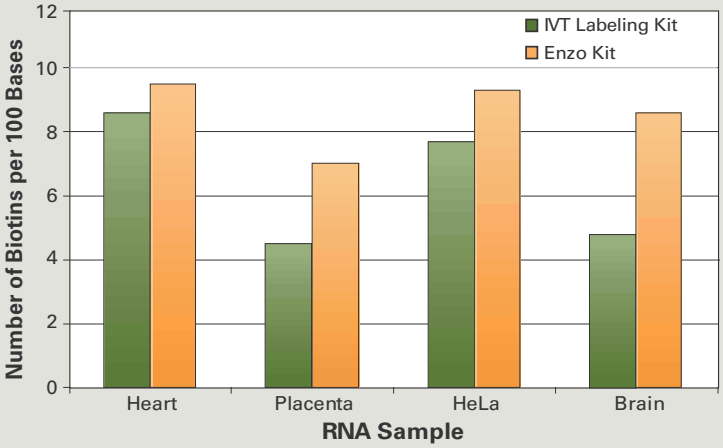


Table 1. Sample array quality metrics from human skeletal muscle total RNA.

Array type	Kit	Ave %P	Background	Noise (RawQ)	Scale Factor	GAPDH-3/5	Actin-3/5
HG-U133 Plus 2.0	IVT 1	35.1%	40	1.10	6.43	0.91	0.92
	IVT 2	35.9%	39	1.09	5.45	0.89	0.99
	Enzo	32.8%	45	1.27	3.15	0.94	0.82
HG-U133A	IVT 1	42.5%	51	1.64	5.00	0.95	0.68
	IVT 2	41.0%	46	1.60	5.88	0.96	0.64
	Enzo	39.9%	41	1.36	3.70	0.92	0.58

As shown in Table 1, the average percentage of Present calls (%P) was similar or slightly higher for the IVT Labeling Kit than for the Enzo Kit. This increase in overall detection sensitivity was more prominent on the 11-µm HG-U133 Plus 2.0 Array, since the hybridization and wash protocols for the IVT Labeling Kit were specifically optimized for the new smaller feature size arrays.

Other metrics, such as background, noise, 3'/5' ratios of control genes, GAPDH, and β-actin, showed a strong similarity between the two labeling methods. Reduced average signal intensities and slightly higher scaling factor values were observed with the new IVT Labeling Kit as compared to the previously recommended Enzo Kit.

Another global array performance parameter, the overall signal correlation, was then analyzed for inter-assay and intra-assay comparisons. Figure 5 shows scatter plot correlations of the Enzo Kit versus the IVT Labeling Kit for the skeletal muscle sample described above on both HG-U133A (Figure 5A) and HG-U133 Plus 2.0 Arrays (Figure 5B). Signal correlations (R^2) ranged from 0.92-0.96 for inter-assay comparisons.

The intra-assay analysis was also performed between the two different lots of IVT Labeling Kit. An R^2 value of greater than 0.99 was obtained on both types of arrays (Figures 5C and 5D), demonstrating good lot-to-lot reproducibility.

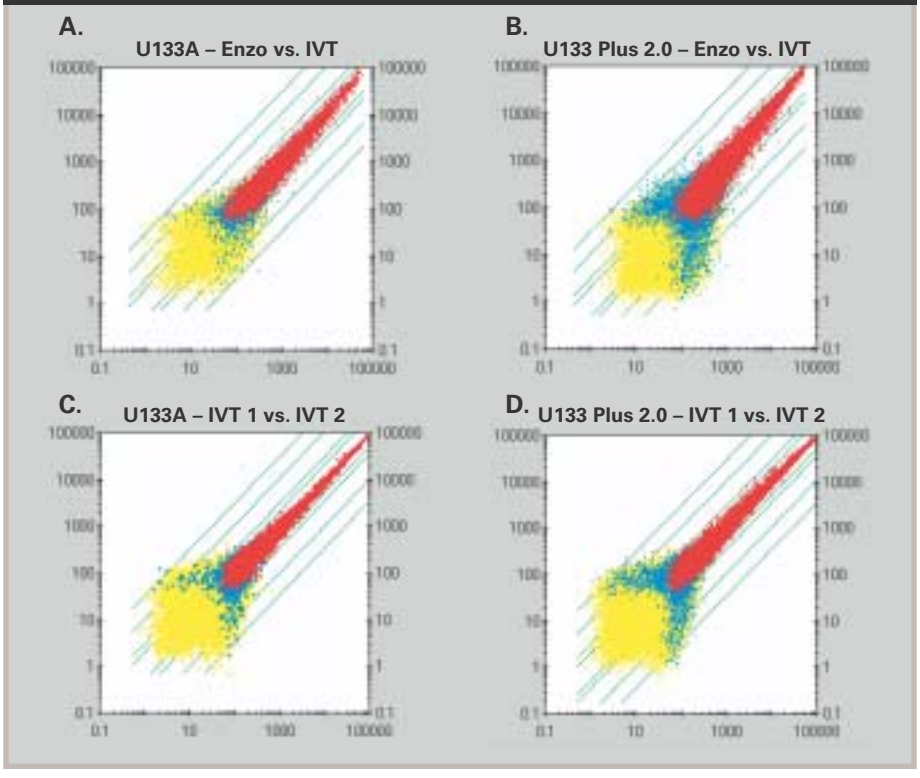
ASSAY SENSITIVITY AND SPECIFICITY

The assay sensitivity and specificity of the new IVT Labeling Kit were assessed and compared with the Enzo Kit in a series of spike-in experiments. Forty-six mouse clones were used to generate cRNA targets that were added to a background sample prepared from D2N cells. All of the spikes had been previously determined to be absent in the background total RNA sample by quantitative RT-PCR. Hybridizations were done in a Latin-Square format utilizing spike concentrations of 0, 0.19, 0.38, 0.75, 1.5, and 3.0 pM.

Following hybridization of the targets to Mouse Genome 430 2.0 Arrays, data were analyzed using Affymetrix® Microarray Suite (MAS) 5.0 statistical algorithms and plotted as ROC curves. Figure 6 shows the Detection call sensitivity and specificity for 0.38 pM, 0.75 pM, and 1.5 pM spike-in concentrations, approximately one copy per 400,000, 200,000, and 100,000 transcripts, respectively.

Detection call sensitivities of 96 percent and 99 percent were observed for the 0.75 pM and 1.5 pM spike concentrations,

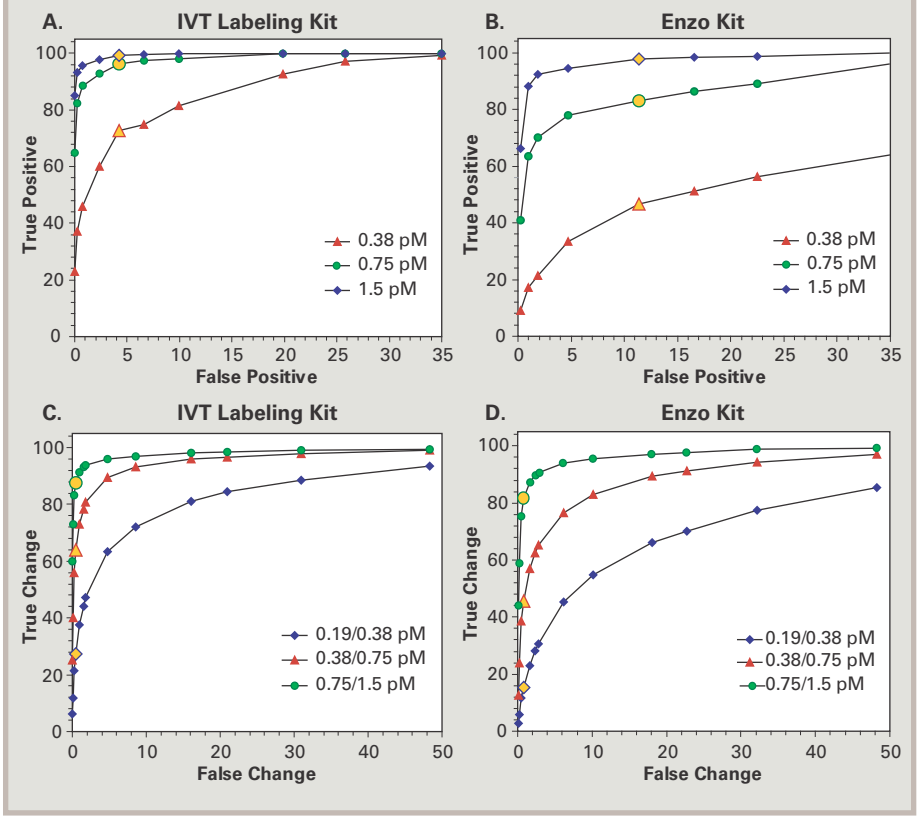
Figure 5. Signal correlation scatter plots for inter-assay and intra-assay comparisons. Targets were prepared from a human skeletal muscle total RNA sample with either the new IVT Labeling Kit or the previously recommended Enzo Kit and hybridized to HG-U133A and HG-U133 Plus 2.0 arrays. Figures 5A and 5B show the signal correlation of inter-assay analysis comparing the Enzo and IVT Labeling Kits on the two different array types. Figures 5C and 5D are intra-assay analyses of two different lots of the IVT Labeling Kit hybridized to either the HG-U133A Array (5C) or the HG-U133 Plus 2.0 Array (5D).



respectively, using the default alpha 1 value in the MAS 5.0 statistical algorithms for samples prepared with the new IVT Labeling Kit (Figure 6A). In both cases, a high specificity of 95 percent was obtained. With the previously recommended Enzo Kit, the Detection call sensitivities were found to be 82 percent and 98 percent with specificities of approximately 90 percent for 0.75 and 1.5 pM spikes, respectively (Figure 6B). This improvement in both sensitivity and specificity with the new labeling reagents was also consistently observed with the lower spike concentration of 0.38 pM (Figure 6).

The data for the Change call analysis are shown in Figures 6C and 6D. In this experiment, the accuracy in detecting a two-fold increase in spike concentration was measured starting at an initial concentration of 0.19 pM, 0.38 pM or 0.75 pM. For targets prepared with the new IVT Labeling Kit, using the default gamma 1 value in the MAS 5.0 statistical algorithms, Change call sensitivities of 64 percent and 88 percent were observed at 0.38 pM or 0.75 pM baseline concentrations, respectively, whereas specificities of 99.5 percent were seen with both pairs of concentration comparisons (Figure 6C). For the Enzo Kit, under the same conditions, reduced Change call sensitivities of 46 percent and 82 percent were obtained at 0.38 pM and 0.75 pM baseline concentrations, respectively, with specificities of 99.2 percent for both cases (Figure 6D). These results were confirmed with the 0.19 pM baseline concentrations. In this analysis, improvement in sensitivity and specificity was observed with the new assay.

Figure 6. Sensitivity and specificity ROC curve analysis of a Latin Square experiment design comparing the two labeling kits. Forty-six endogenous mouse transcripts were spiked into the D2N cell line background at various concentrations. This cell line had been previously shown to be void of these transcripts by quantitative RT-PCR. Detection sensitivity (true positive rate) is plotted on the y axis versus specificity (1 minus false positive rate) on the x axis. **A and B.** Detection ROC curves at various spike concentrations (0.38 pM, 0.75 pM, and 1.5 pM) are shown. Samples were prepared using the new IVT Labeling Kit (Figure 6A) or with the Enzo Kit (Figure 6B) and hybridized to Mouse 430 2.0 arrays. Results show increased sensitivity and specificity for the new labeling reagents at each respective concentration of spikes. Enlarged data points indicate sensitivity/specificity at the default alpha 1 value for the MAS 5.0 statistical algorithms. **C and D.** Change call ROC curves indicate the likelihood of making a correct Change call with the MAS 5.0 statistical algorithms across various two-fold spike concentrations. Results show similar Change call sensitivity between the IVT Labeling Kit and Enzo Kit at 0.75/1.5 pM comparisons but improved sensitivity for the new IVT Labeling Kit at lower spike concentration comparisons. Change call specificity is slightly better for the IVT Labeling Kit. Enlarged data points indicate sensitivity/specificity at the default gamma 1 value for the MAS 5.0 statistical algorithms.

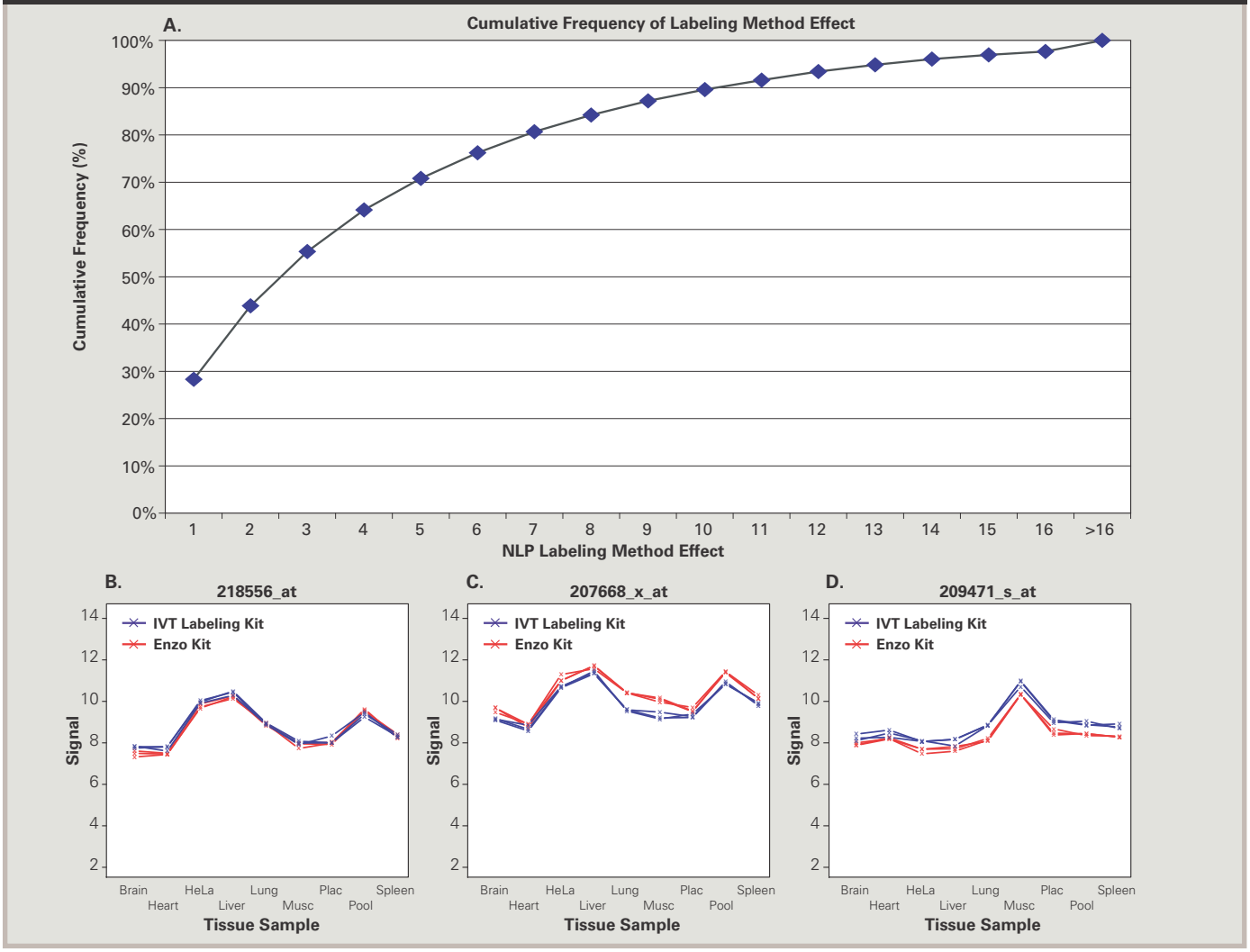


PROBE SET SIGNAL ANALYSIS

In order to assess the existence of any systematic differences in array results between the two labeling reagents, a two-way ANOVA was performed on a panel of human tissue and cell line samples. Nine RNA samples were labeled with the new IVT Labeling Kit and the Enzo Kit; each target sample was hybridized to HG-U133 Plus 2.0 Arrays in triplicate.

For each of 54,674 probe sets on the HG-U133 Plus 2.0 Array, a two-way ANOVA model was applied to quantile-normalized signal estimates. One factor was the labeling method (the IVT Labeling Kit or the Enzo Kit) and associated hybridization and wash conditions and the other factor was sample (the nine different tissue samples used). At a significance level of 10^{-3}, the null hypothesis of no difference in signal due to labeling method was rejected 45 percent of the time, indicating that for these probe sets, the signal values for the same probe set in different assays are not always directly comparable (Figure 7).

Figure 7. Two-Way ANOVA for probe set level signal comparison of the two labeling methods. The analysis was performed using labeling method and tissue diversity as the two factors. **A.** The plot is based on *p*-values corresponding to tests of the null hypothesis that the labeling methods perform the same across the tissues. Negative $\log_{10}$ *p*-values (NLP) are plotted as a cumulative frequency histogram. Higher NLP values correspond to statistically more significant differences between the two labeling methods. **B.** Example of a probe set showing no statistically significant difference between labeling methods (NLP=0.06). Log_2 (Signal) is plotted on the y axis as a function of tissues on the x axis. The triplicate array Signal values are plotted for each labeling method (red for the Enzo Kit, blue for the IVT Labeling Kit). **C.** Example of a probe set showing a significant difference between labeling methods (NLP=6). **D.** Example of a probe set showing highly significant difference between labeling methods (NLP=17). Note that despite differences in labeling methods, the behavior is consistent between tissues. Therefore, derived quantities such as fold-changes remain unaffected. Note also that the increase or decrease in signal intensities due to labeling method may vary for different probe sets.



However, a statistically significant difference does not necessarily imply a large or important difference, so it is also instructive to look at the contribution of the two different labeling methods to the observed variance in signal estimates. The ANOVA model attributed 81 percent of the observed variation in signal estimates to tissue and only 6 percent to the labeling method and assay. The large proportion of variance attributed to the sample demonstrated the responsiveness of probe sets to different expression levels in different tissues. Only 4 percent of the variation was attributable to a sample-labeling method interaction term and 9 percent of the overall variance was left unexplained by the model. Therefore, although the labeling method led to a significant difference for many of the probe sets, the difference was relatively small compared to the variation in signal estimates from one sample type to another. Results of the ANOVA are shown in Figure 7.

Figures 7B-D present a closer view of the comparison between signal intensities for the two labeling methods across a

Figure 8. Signal log ratio analysis comparing the two different labeling methods. Targets prepared from brain and heart total RNA samples were hybridized to HG-U133 Plus 2.0 Arrays and differential expression fold changes were calculated using the heart total RNA as the baseline sample. Signal log ratios (SLR, log base 2) were plotted for probe sets that were called as Present in both brain and heart samples (10,649 probe sets). There is a high level of correlation between the SLR values obtained from the two different labeling methods.

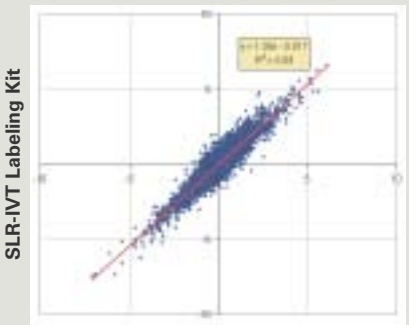
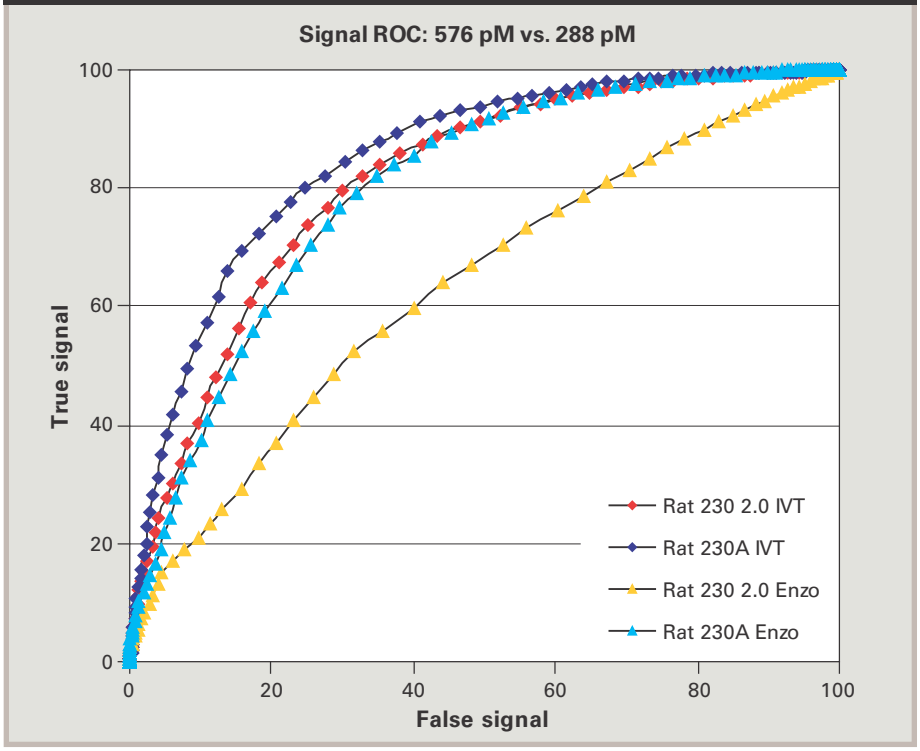


Figure 9. Signal ROC curve analyses of 288 and 576 pM spikes. In a Latin-Square design for each platform, forty-eight pre-labeled exogenous transcripts were spiked into a rat cell line sample (RBL-1 lymphoblast) which was empirically checked by quantitative RT-PCR to be void of these transcripts. For each spike pool, triplicate arrays were hybridized across three independent lots of the respective array designs (Rat Genome 230 2.0 Array or Rat Expression Array 230A) and Signal was calculated using the MAS 5.0 statistical algorithms. True positives (y axis) are successful detection of significant signal change between the two different concentrations. False positives (x axis) are detection of significant signal change between replicates of the same concentration. Significance was defined by a t-like statistic of the difference in Signal divided by the standard deviation of variation across replicates.



range of typical probe sets. Note that even when a significant labeling method effect was found, the size of the effect was generally the same across all samples. The implication is that when looking at fold-changes or other comparative metrics, the result is expected to be the same, regardless of which labeling method is used.

To further explore the similarity or difference between signal intensity at the probe set level, differential expression calls were compared between a brain and heart sample on HG-U133 Plus 2.0 Arrays. When filtered for probe sets called as Present in both tissues, the signal log ratios (SLR) showed a high degree of correlation (R^2 = 0.88) between samples labeled with the IVT Labeling Kit and the Enzo Kit (Figure 8). Within-method SLR correlations using the same parameters showed just slightly better correlations with R^2 = 0.90 (data not shown).

In summary, although a majority of the probe sets shows a high degree of similarity between the two different labeling methods, differences in signals do exist for a number of probe sets.

ASSESSMENT OF SIGNAL SATURATION FOR HIGH CONCENTRATION TRANSCRIPTS

Previous studies indicated that when using the Enzo Kit to prepare targets for hybridization to the 11-µm HG-U133 Plus 2.0 Array, signal intensities for transcripts expressed at very high levels showed some degree of saturation compared with data obtained on the 18-µm HG-U133A Array. The potential of the IVT Labeling Kit to help alleviate this high-end saturation effect was investigated. Two high-concentration spikes, at 288 and 576 pM, were used on the Rat 230A and Rat 230 2.0 Arrays with targets prepared with either the Enzo Kit or the new IVT Labeling Kit.

The Rat 230 2.0 Array, when used with the Enzo Kit, showed significantly reduced sensitivity and specificity compared to the Rat 230A Array, replicating the high-end saturation described previously (Figure 9). In contrast, samples prepared with the new IVT Labeling Kit provided similar sensitivity and specificity profiles between the two array types. The high-concentration signal saturation observed with the previously recommended Enzo Kit on the 11-µm feature arrays was significantly reduced when using the new IVT Labeling Kit.

Conclusion

The GeneChip® Expression 3'-Amplification Reagents for IVT Labeling were shown to produce consistently high cRNA yields over a wide range of samples. The new labeling reagents are robust and can generate sufficient targets from starting samples of as low as 1 µg of total RNA. In addition, the IVT Labeling Kit was also demonstrated to be compatible with the Two-Cycle Target Labeling Assay, although the data are not presented in this Technical Note.

Even though the new kit utilizes a single label, results generated from this new kit were able to confirm most of the observations made using the previously recommended two-label formulation in the Enzo Kit. However, for researchers with large data sets, the question of direct comparability of data between the two labeling methods should be assessed individually, based on the goals of the study and the analytical techniques that will be used, since differences at the signal level exist for a number of probe sets.

Spike-in experiments showed that the new IVT Labeling Kit produced better sensitivity and improved specificity for both Detection and Change calls, especially for transcripts present at relatively low concentrations. This resulted from the optimized hybridization and wash conditions with the new 11-µm array platform. With the more streamlined protocol and more convenient packaging options, the new GeneChip IVT Labeling Kit will be the labeling method of choice for expression analysis on GeneChip arrays.

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