GeneChip® Expression 3’-Amplification Reagents for IVT Labeling

Optimized Protocol for
Improved Results

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Outline

- Overview
- Technical performance characterization
  - cRNA yield and lengths
  - Basic array metrics
  - Detection and Change call sensitivity and specificity
  - Probe Set Signal analysis
  - Assessment of signal saturation for high concentration transcripts
- Summary

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A Complete Reagent Solution for GeneChip® Expression Analysis

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- Target labeling and control reagents specific for GeneChip expression applications
- The High-Resolution Scanner, new Fluidics Station, 11-μm arrays, and new reagents represent the next generation system offering the best performance
- Support both One- and Two-Cycle Target Labeling Assays
- Provide consistency, convenience, ease of use, and standardization

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GeneChip® Target Labeling Assays

Starting Materials		Target Labeling Assay
Total RNA	mRNA	
1 – 15 μg	0.2 – 2 μg	One-Cycle
10 – 100 ng	N/A	Two-Cycle

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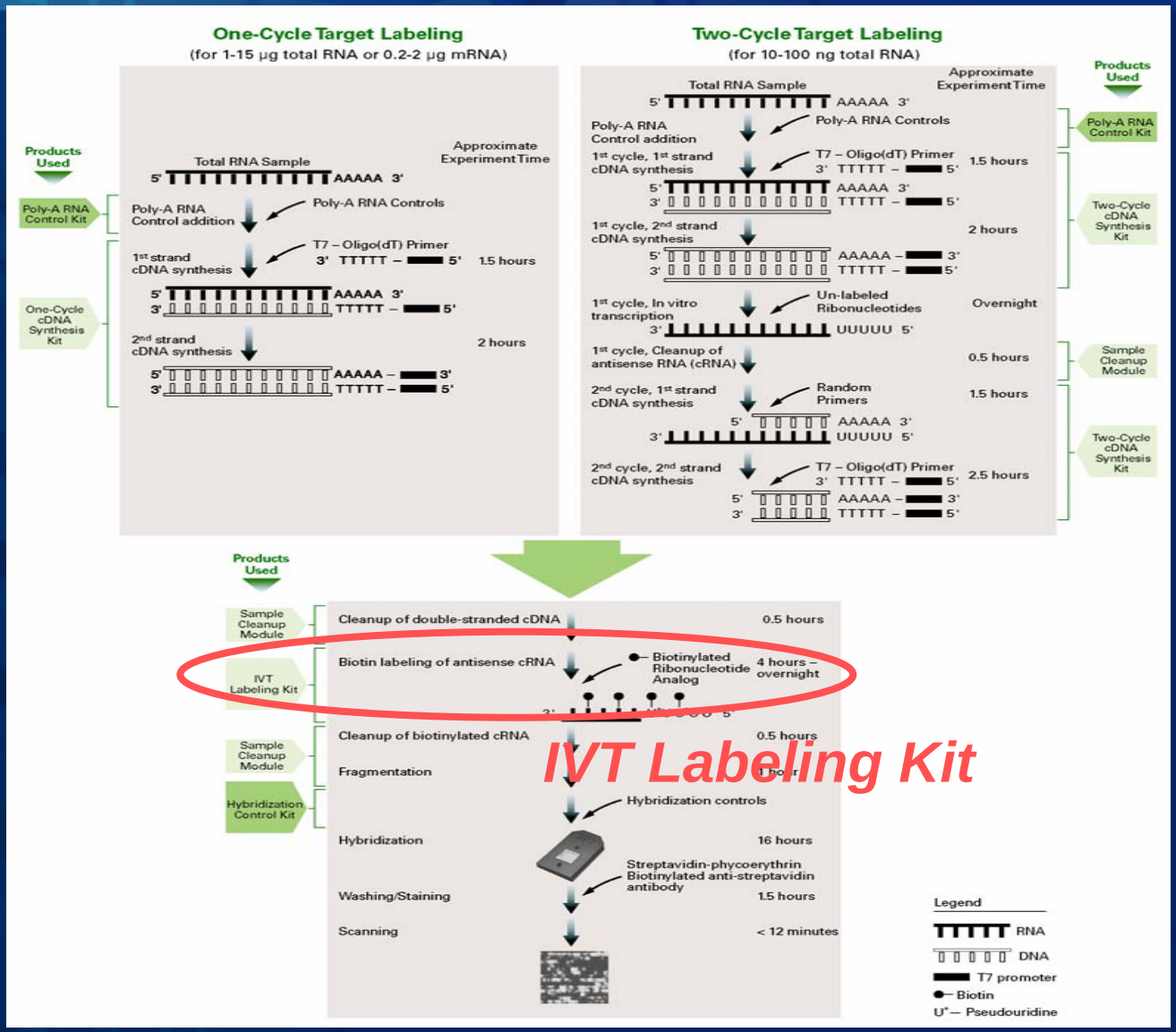
GeneChip® Target Labeling Assays and Reagents



IVT Labeling Kit

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One-Cycle Target Labeling and Control Reagents

One-Cycle Target Labeling and Control Reagents (30 reactions) P/N 900493		
One-Cycle cDNA Synthesis Kit	1	900431
IVT Labeling Kit	1	900449
Sample Cleanup Module	1	900371
Poly-A RNA Control Kit	1	900433
Hybridization Controls	1	900454

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Two-Cycle Target Labeling and Control Reagents

Two-Cycle Target Labeling and Control Reagents (30 reactions) P/N 900494		
Two-Cycle cDNA Synthesis Kit	1	900432
IVT Labeling Kit	1	900449
Sample Cleanup Module	2	900371
Poly-A RNA Control Kit	1	900433
Hybridization Controls	1	900454

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GeneChip® IVT Labeling Kit

Component Name	Volume
10X IVT Labeling Buffer	120 µL
IVT Labeling Enzyme Mix	120 µL
IVT Labeling NTP Mix	360 µL
3'-Labeling Control (0.5 µg/µL)	10 µL
RNase-free Water	910 µL

- 30 reactions/kit
- *in vitro* transcription with MEGAscript® Reagents
- Shelf life 12 months

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IVT Labeling Kit Features

- Consistent yield and incorporation efficiency, from as low as 1 μg of total RNA as starting material with the One-Cycle Target Labeling Assay
- Developed as part of the new 2.0 Platform (11-μm arrays)
- Increased sensitivity and improved specificity over the previously recommended protocol
- Quality tested and standardized for GeneChip platform
- Streamlined experimental flow
- Convenient packaging

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Optimized Protocol

Labeling	Single label with a proprietary pseudouridine reagent
IVT incubation	16 hours with an optional 4 hour-incubation protocol
Starting material	1 – 15 μg of total RNA for the One-Cycle Target Labeling Assay
Hybridization	Including 10% DMSO in cocktail
Washing & Staining	More stringent Fluidics Protocol for 11-μm, 49- and 64- format arrays

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IVT Labeling Kit Technical Note

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AFFYMETRIX® PRODUCT FAMILY > RNA ARRAYS AND REAGENTS >

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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 10X IVT Labeling 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).

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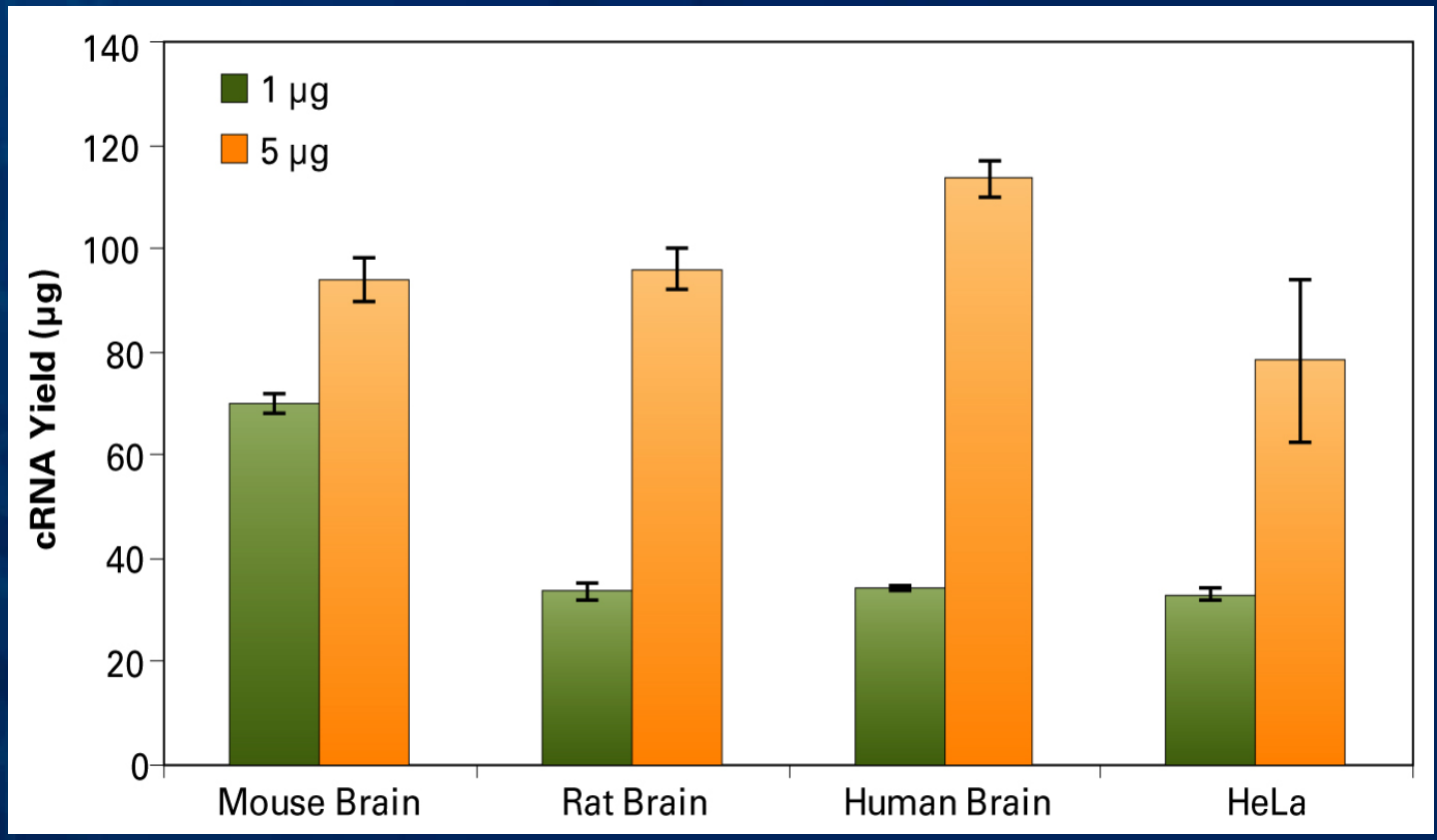
High cRNA Yield From as Low as 1 μg Total RNA in the One-Cycle Target Labeling Assay



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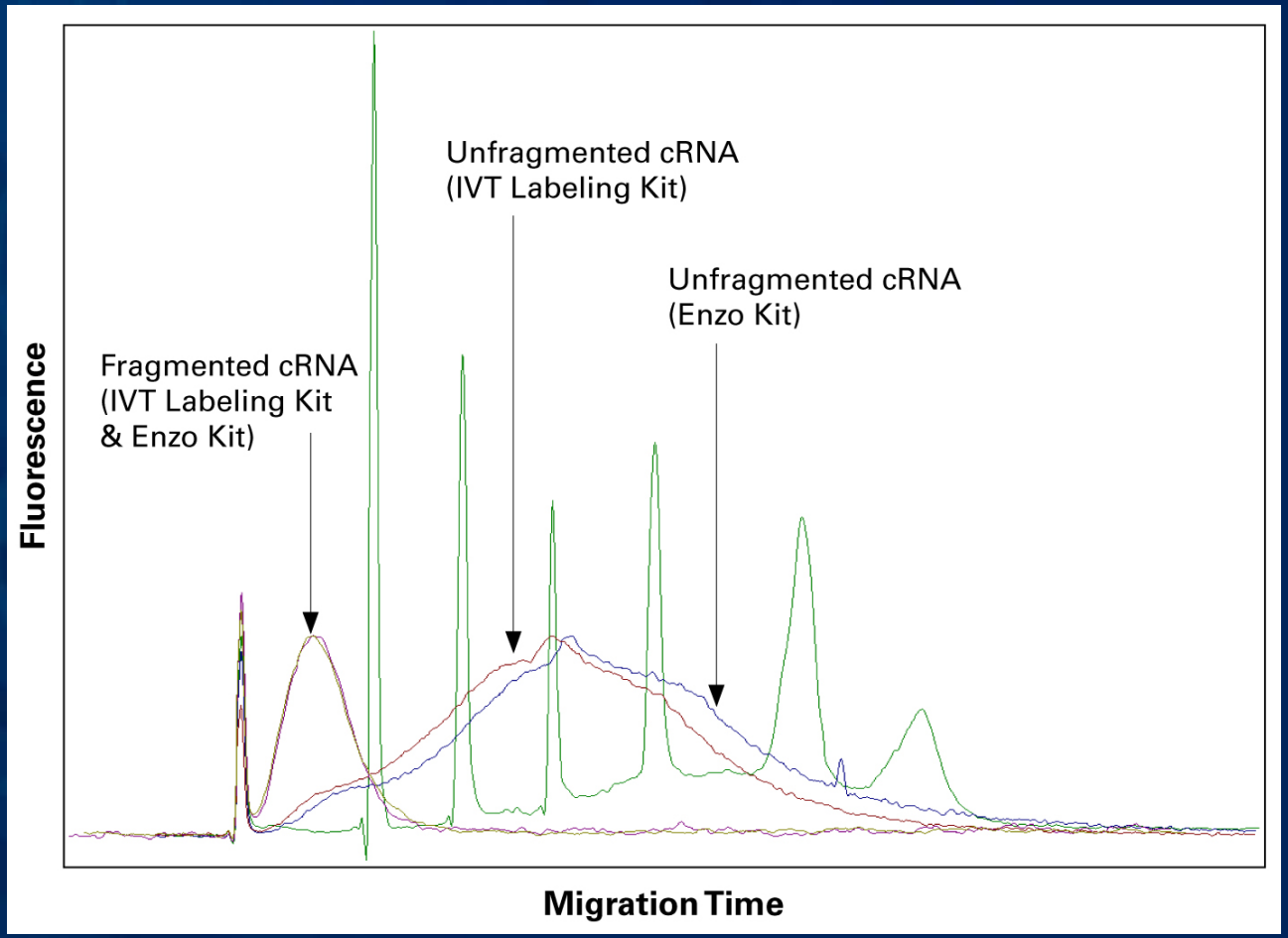
cRNA Length Comparing Labeling Methods



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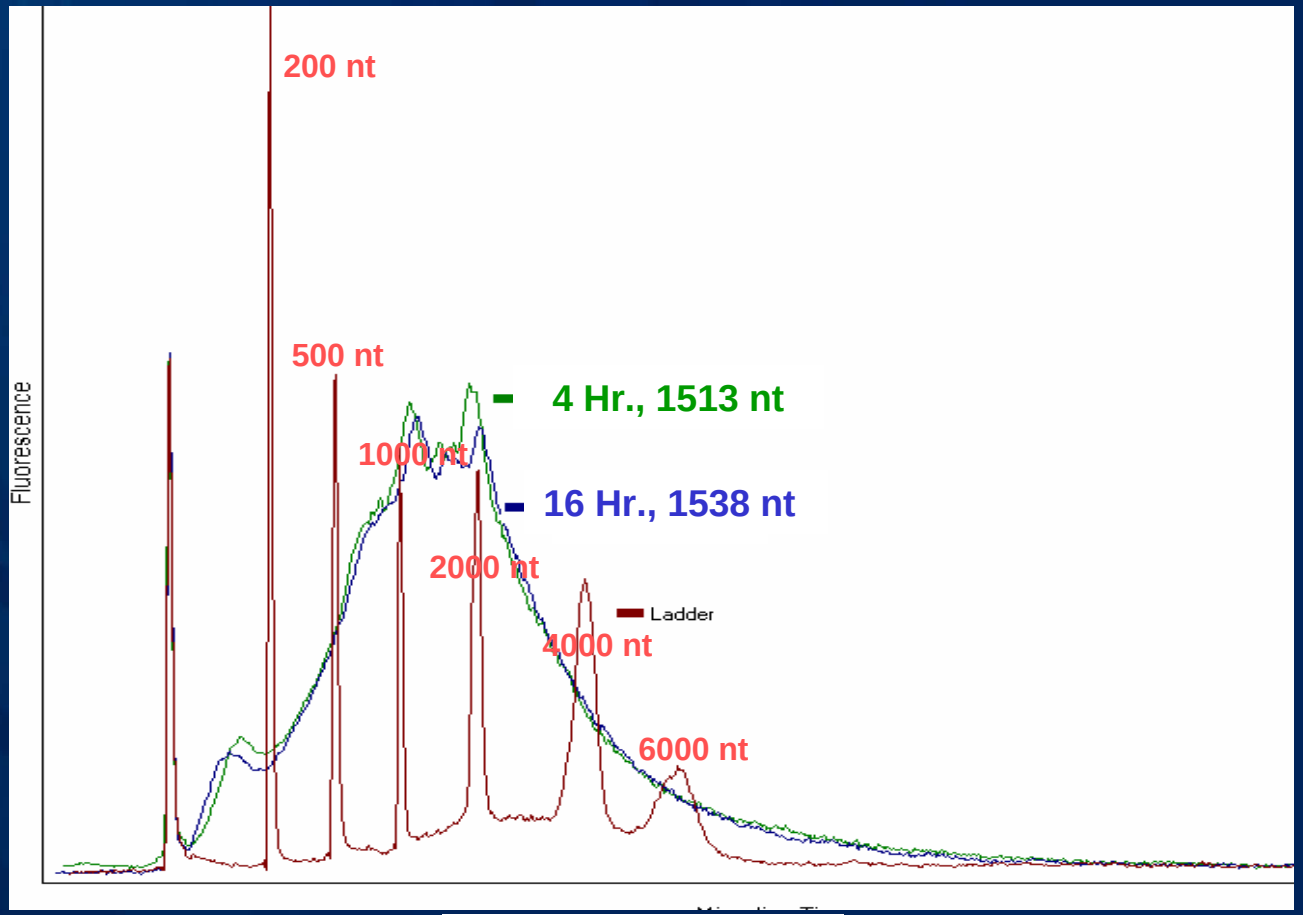
Overnight Incubation Does Not Affect cRNA Length



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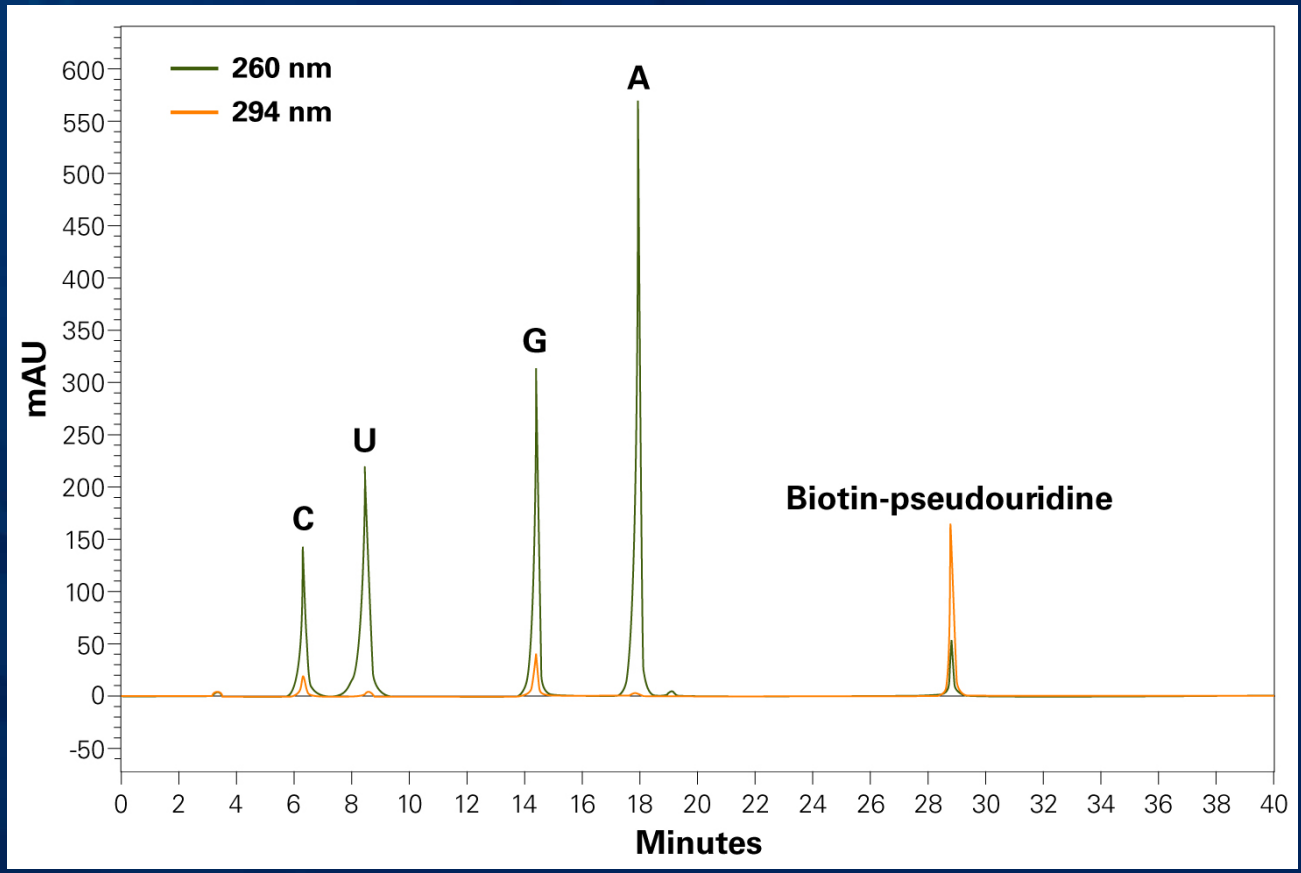
HPLC Trace for Biotin Incorporation Quantitation



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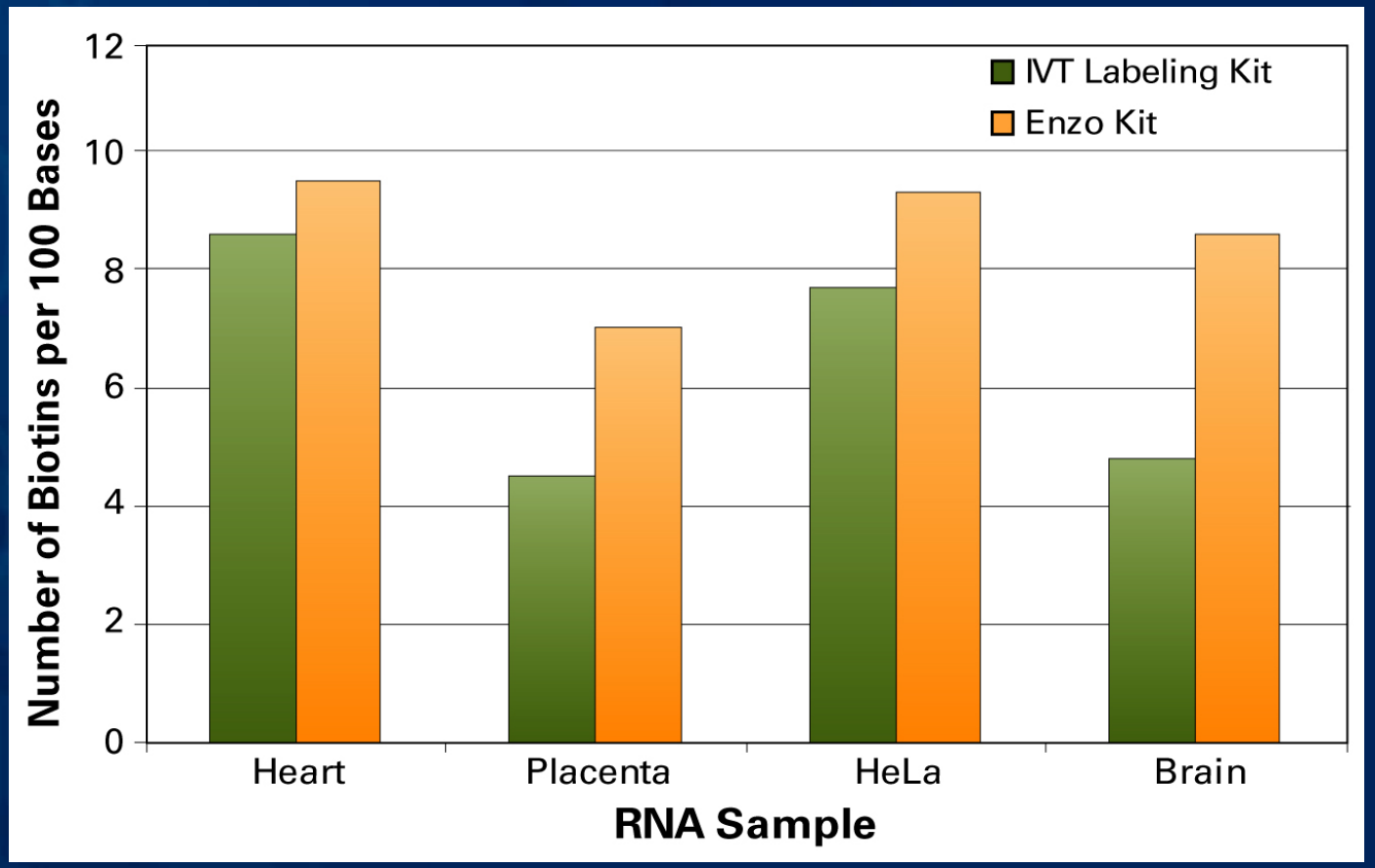
Biotin Incorporation Rates of Two Labeling Methods



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Basic Array Quality Metrics

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

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Basic Array Metrics

	IVT	Amount	cRNA yield (ug)
HeLa	Enzo	1 ug	32
		5 ug	29
	IVT	1 ug	33
		5 ug	78
Brain	Enzo	1 ug	28
		5 ug	38
	IVT	1 ug	34
		5 ug	114

	IVT	Amount	% Present calls	3'/5' GAPDH	3'/5' Actin
HeLa	Enzo	1 ug	47.8%	0.94	0.83
		5 ug	47.7%	0.99	0.96
	IVT	1 ug	47.3%	0.90	0.91
		5 ug	49.1%	0.86	1.00
Brain	Enzo	1 ug	56.3%	1.27	1.73
		5 ug	52.8%	1.19	1.61
	IVT	1 ug	53.0%	1.51	2.24
		5 ug	53.7%	1.52	2.25

	IVT	Amount	Signal Correlation	
			Intra	Inter E/R
HeLa	Enzo	1 ug	0.989	
		5 ug	0.989	
	IVT	1 ug	0.990	0.957
		5 ug	0.982	0.953
Brain	Enzo	1 ug	0.991	
		5 ug	0.986	
	IVT	1 ug	0.986	0.949
		5 ug	0.983	0.947

	IVT	Amount	Call Concordance	
			Intra	Inter E/R
HeLa	Enzo	1 ug	93.16%	
		5 ug	92.45%	
	V	1 ug	91.30%	90.73%
		5 ug	91.89%	90.08%
Brain	Enzo	1 ug	92.70%	
		5 ug	92.37%	
	IVT	1 ug	89.75%	89.63%
		5 ug	90.84%	89.75%

	IVT	Amount	False Change	
			Intra	Inter E/R
HeLa	Enzo	1 ug	1.75%	
		5 ug	0.48%	
	IVT	1 ug	0.71%	2.39%
		5 ug	0.49%	4.12%
Brain	Enzo	1 ug	0.16%	
		5 ug	0.21%	
	IVT	1 ug	0.46%	1.02%
		5 ug	0.38%	1.68%

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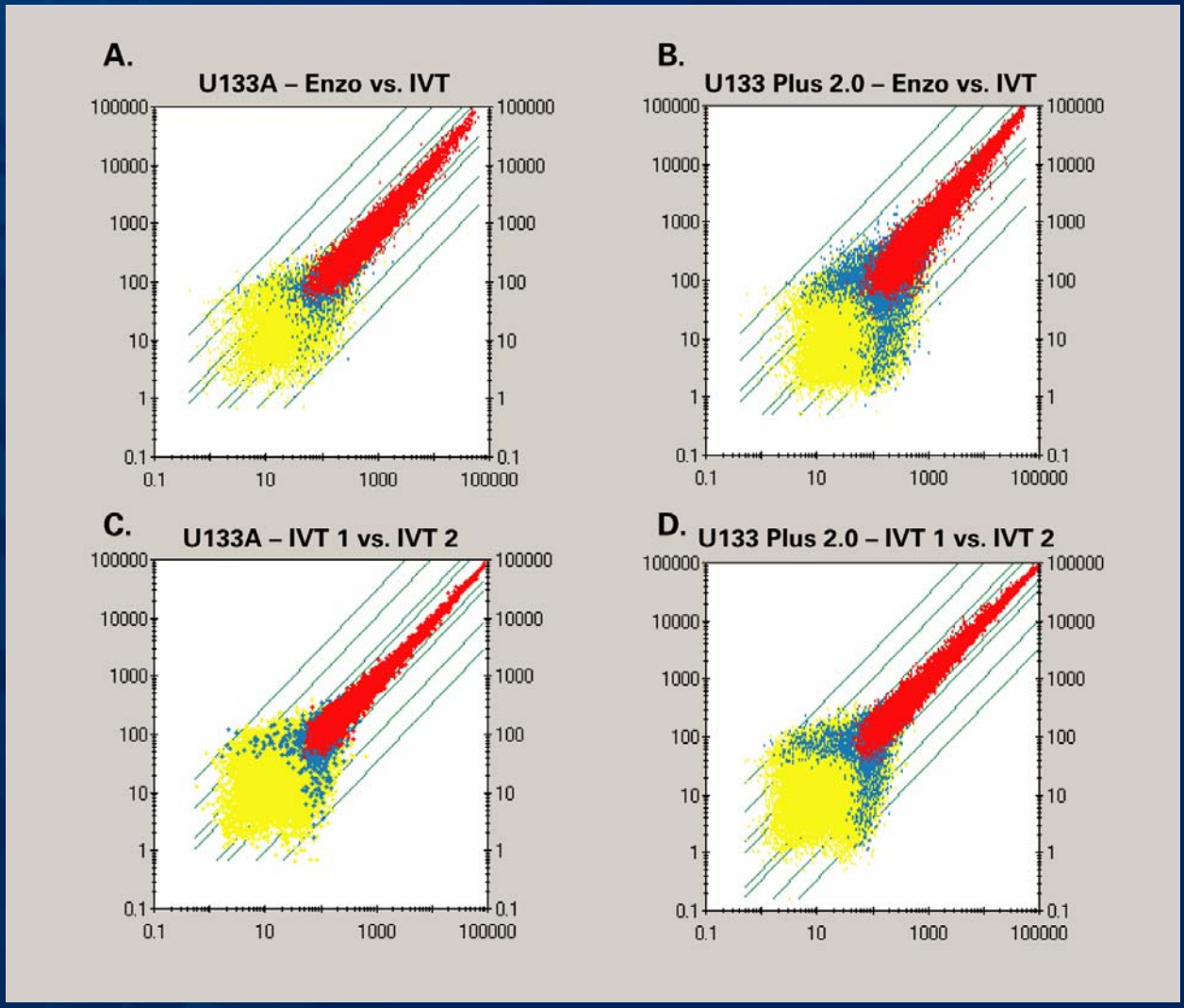
Overall Signal Correlation



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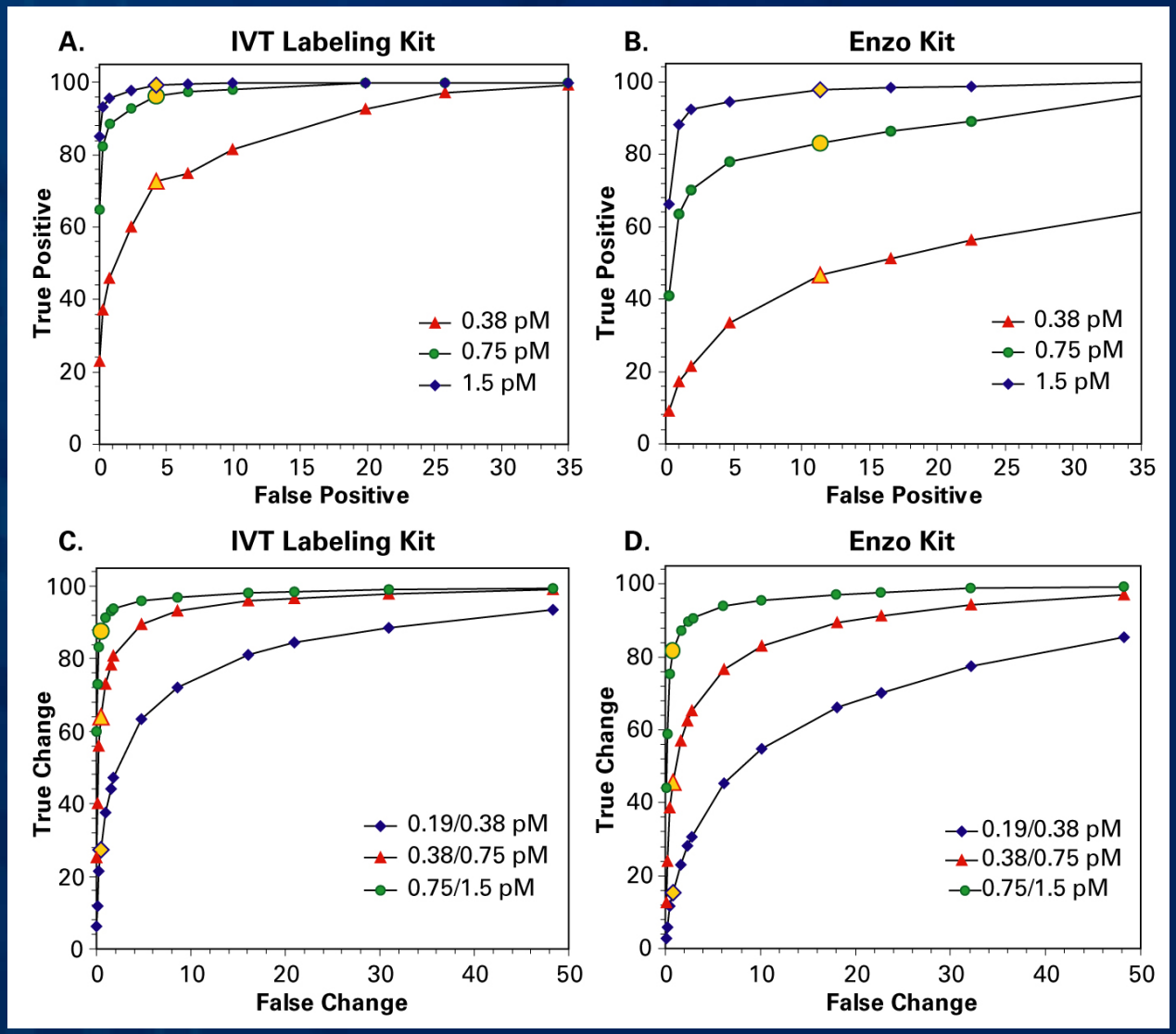
Higher Sensitivity and Specificity with the IVT Labeling Kit



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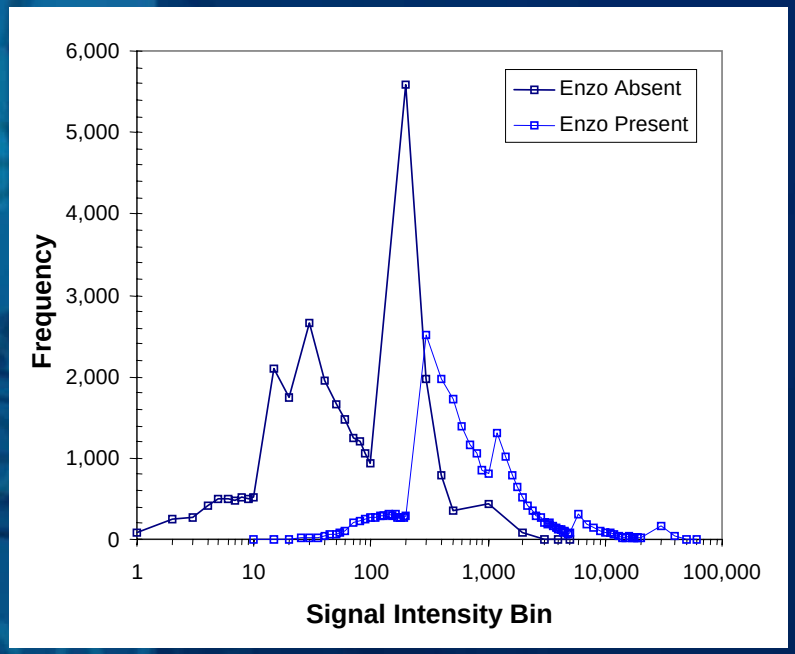
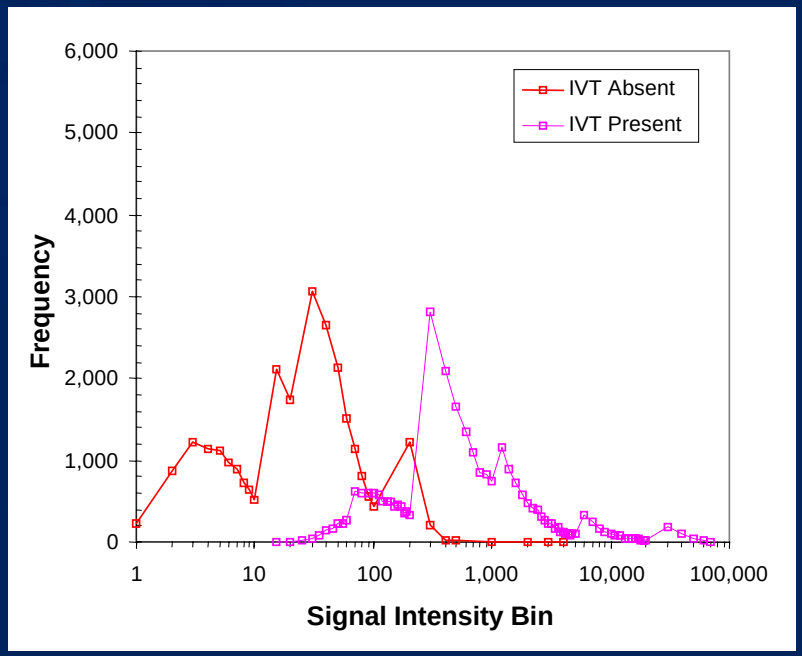
Increased Specificity with the IVT Labeling Kit





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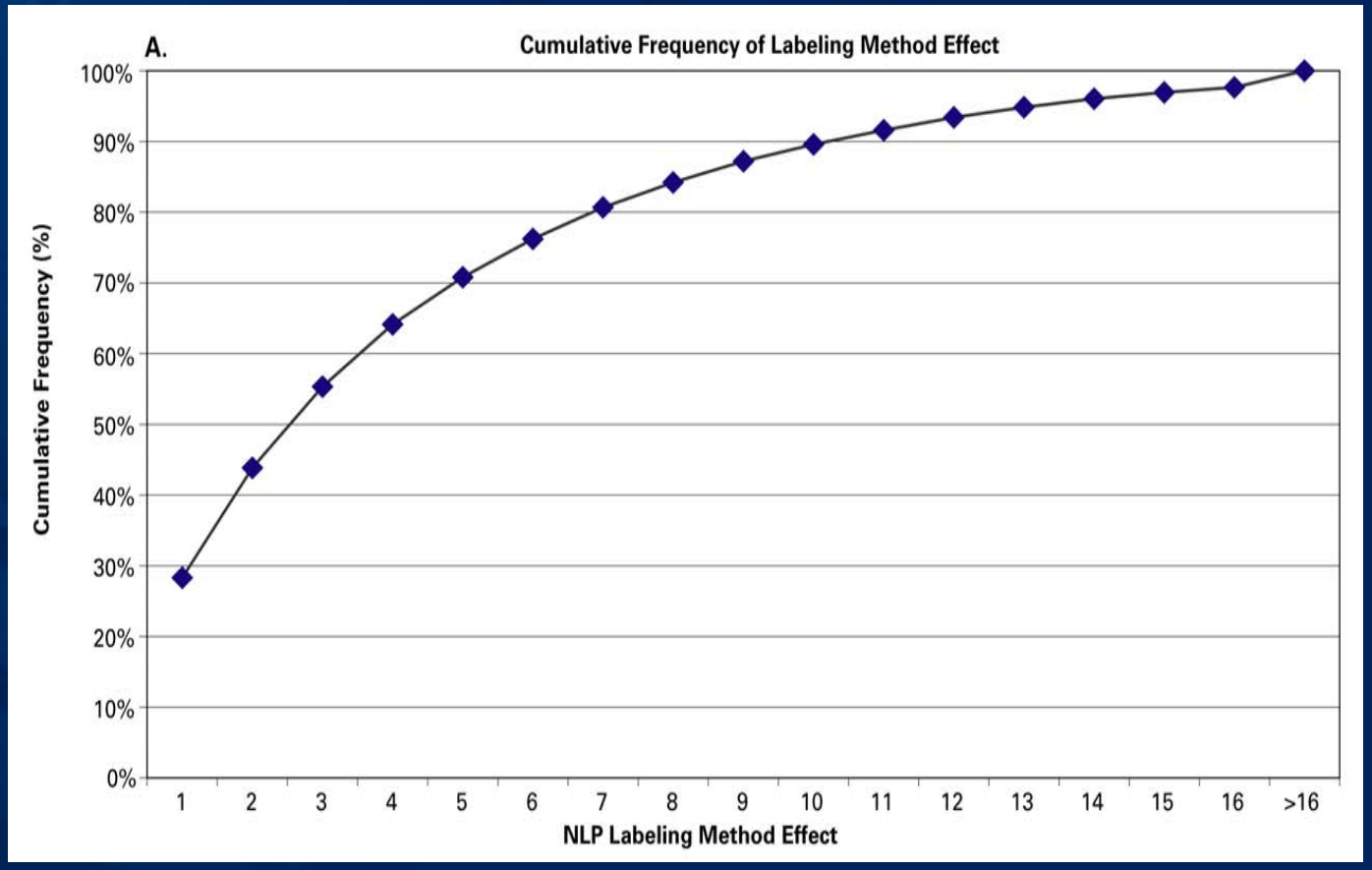
Two-Way ANOVA for Probe Set Signal Comparison



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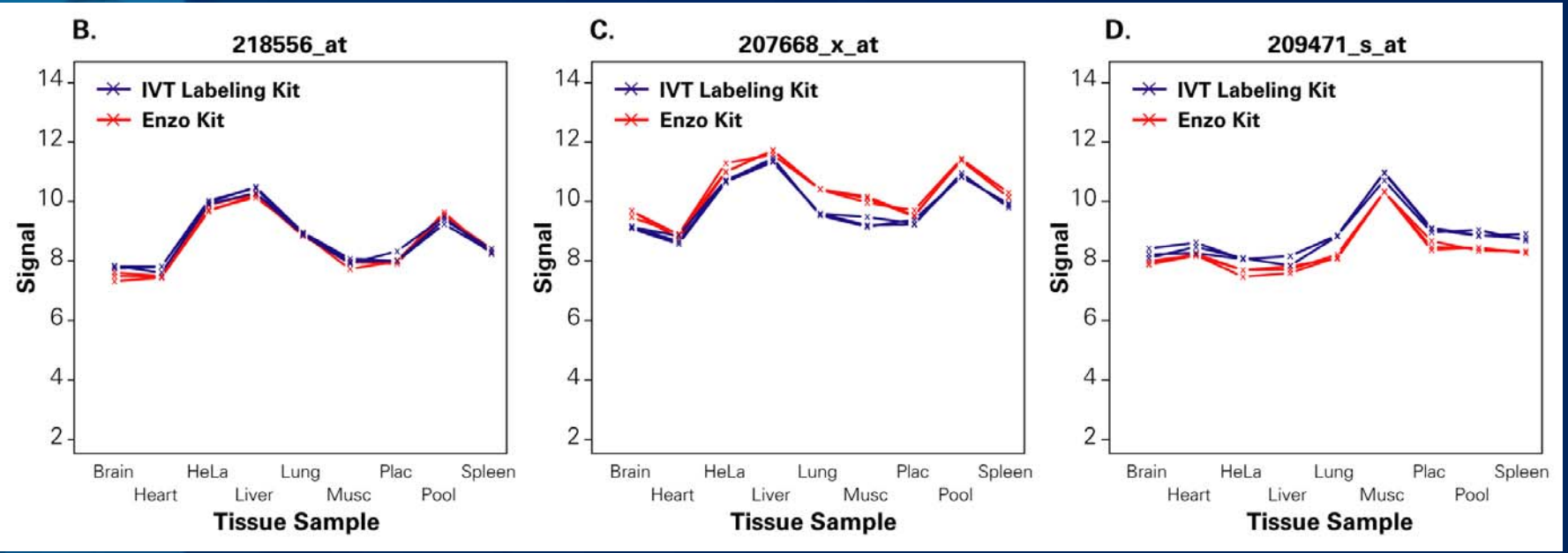
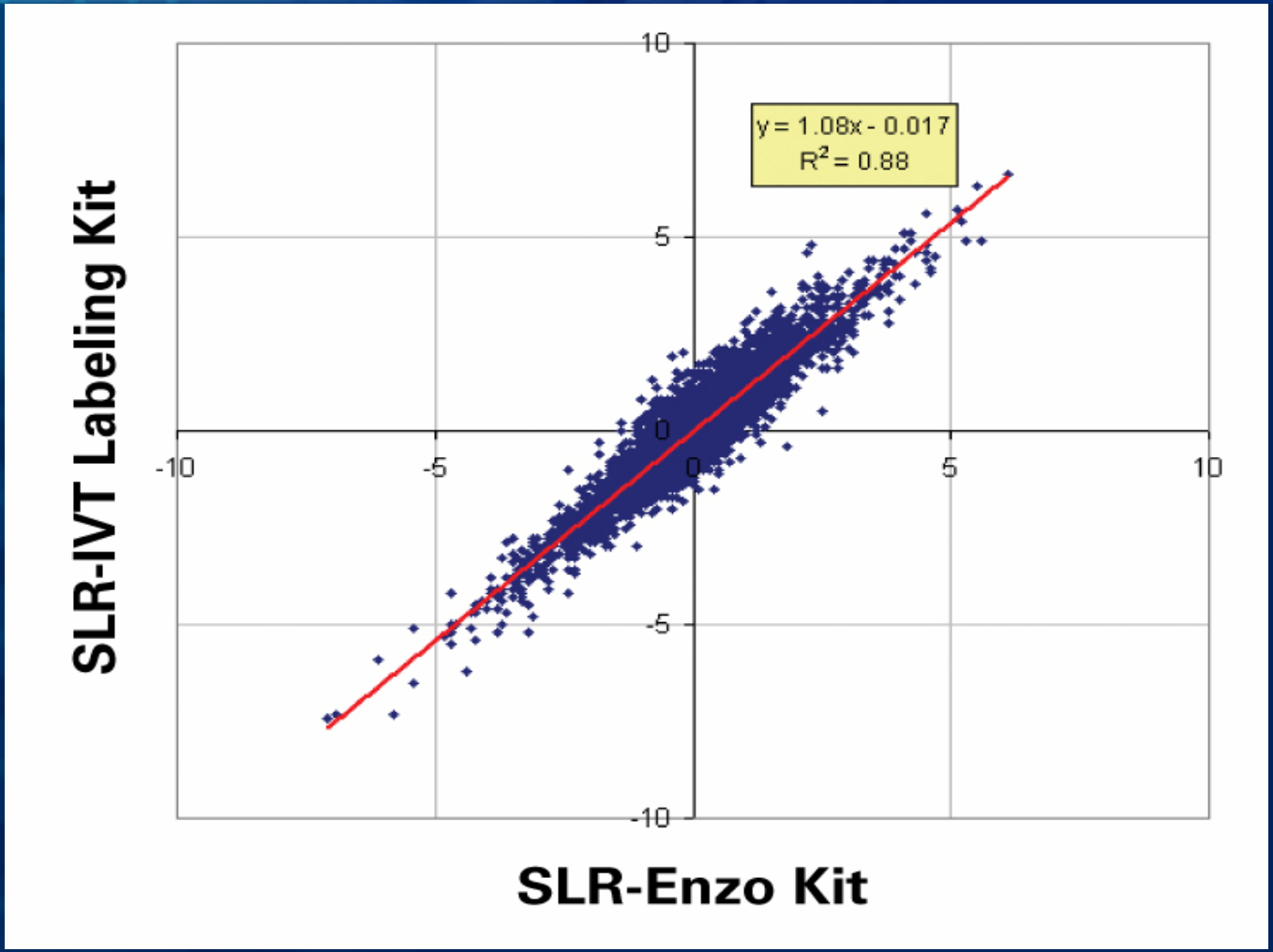
Examples of Probe Set Intensities



Signal Log Ratio Analysis



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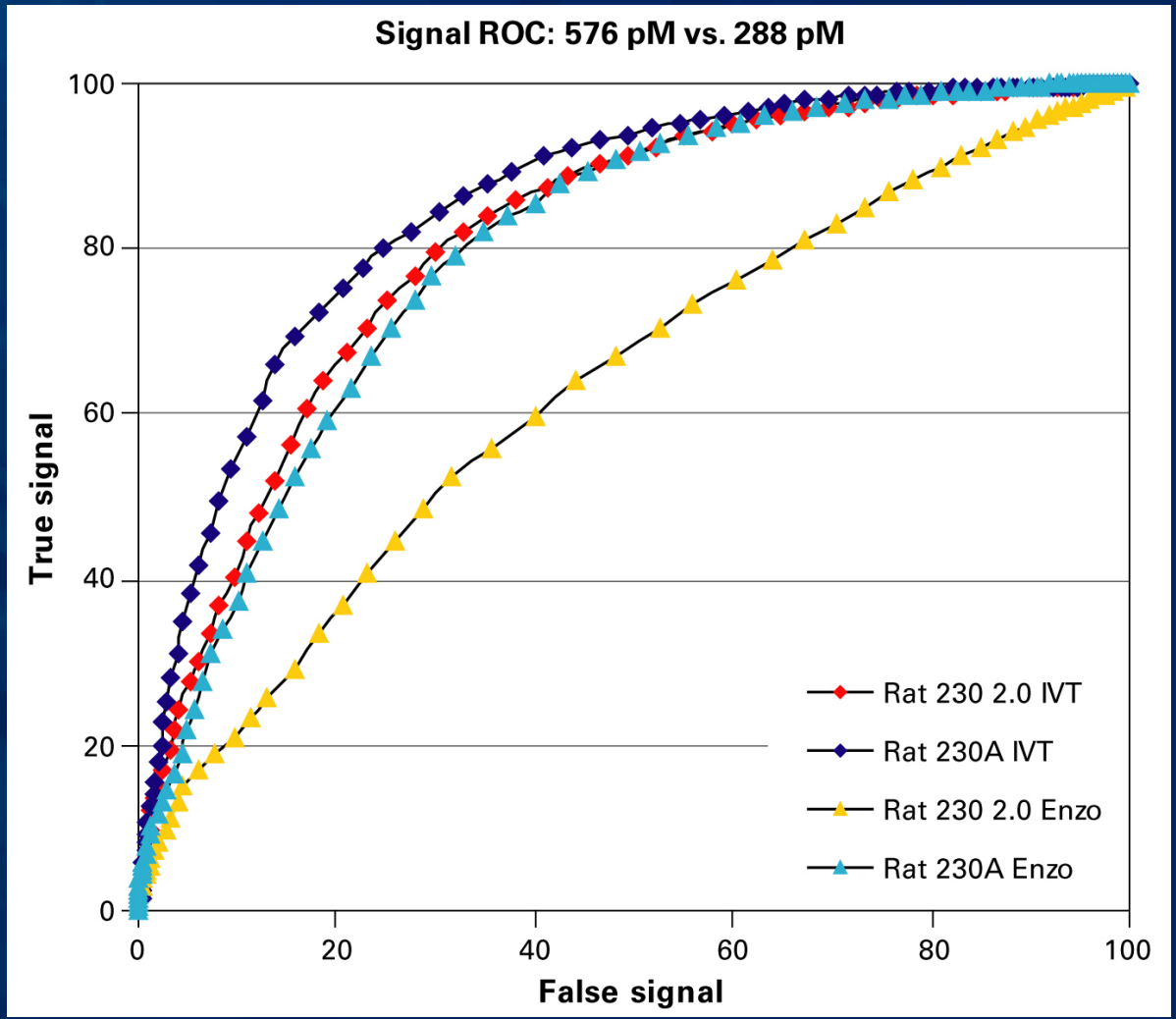
Significantly Reduced Signal Saturation with the New IVT Labeling Kit



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Optimized Protocol for Improved Results

- Robust and consistent high cRNA yields over a wide range of samples
- Optimized hybridization and wash conditions produced better sensitivity and improved specificity for both Detection and Change calls
- Better experimental flow and convenient packaging
- Probe set level signal values do differ for a number of probe sets although the fold-changes or other comparative metrics are expected to be similar between the labeling methods
- Direct comparability of data between two labeling methods should be assessed individually

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