White Paper

GeneChip® 3’ IVT Express Kit

Abstract

The GeneChip® 3’ IVT Express Kit is the newest option for generating target for Affymetrix 3’ expression arrays. The 3’ IVT Express Kit utilizes the same basic chemistry as GeneChip® One-Cycle Target Labeling and Control Reagents, but employs a simplified workflow and optimized reagents that enable lower RNA input and increased ease of use. In this white paper, we describe the technical performance of the 3’ IVT Express Kit and compare array results with One-Cycle Reagents.

Introduction

Affymetrix 3’ expression arrays have probe sets targeted to the 3’ end of the transcript. Target for these arrays is prepared using amplification and labeling methodologies that generate labeled target initially primed from the poly-A tail of the transcript. First-strand cDNA is synthesized using an oligo-dT primer with an attached T7 promoter sequence. After making the complementary second strand, the double-stranded cDNA molecule is amplified in a linear fashion by in vitro transcription (IVT) using T7 RNA polymerase. As the aRNA (amplified RNA, also commonly referred to as cRNA) is being made, a biotinylated nucleotide analog is incorporated and serves as a label for the message. After fragmentation, this labeled anti-sense aRNA is hybridized to the array.

We have developed a new kit that utilizes this same basic strategy for generating target for hybridization to GeneChip® 3’ expression arrays. The 3’ IVT Express Kit uses optimized reagents that dramatically reduce the total RNA input requirements. For most RNA types, 50 to 100 ng of total RNA will generate sufficient amounts of labeled target for the largest-format cartridge array. Because of the low total RNA input requirements, the 3’ IVT Express Kit is intended to replace the One-Cycle and Two-Cycle Target Labeling and Control Reagents.

In addition, the 3’ IVT Express Kit provides a simplified workflow and includes pre-made master mixes that decrease hands-on time (see Figure 1). Those using higher input amounts (100 to 500 ng) can complete the target preparation protocol in a single day. Compared to One-Cycle Reagents, the cDNA cleanup step has been removed and all purification steps now use magnetic beads instead of spin columns for enhanced recovery. The kit contains all of the reagents needed to generate labeled aRNA target and also includes a control RNA sample, poly-A RNA and hybridization controls, and the plastic consumables needed to run the assay.

Figure 1: Workflow comparison of 3′ IVT Express Kit to One- and Two-Cycle Reagents.

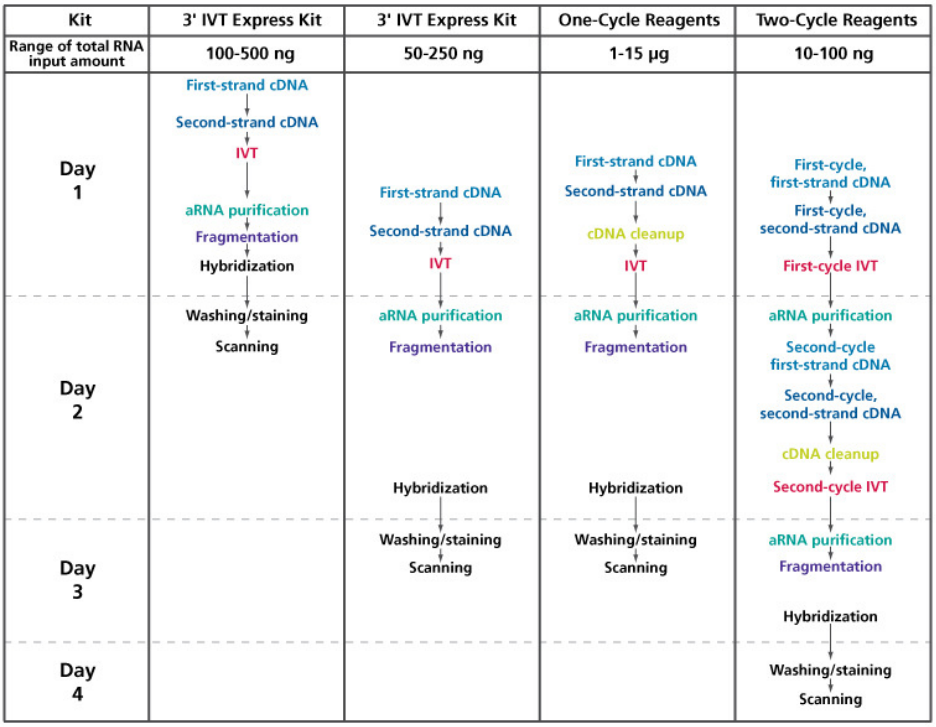
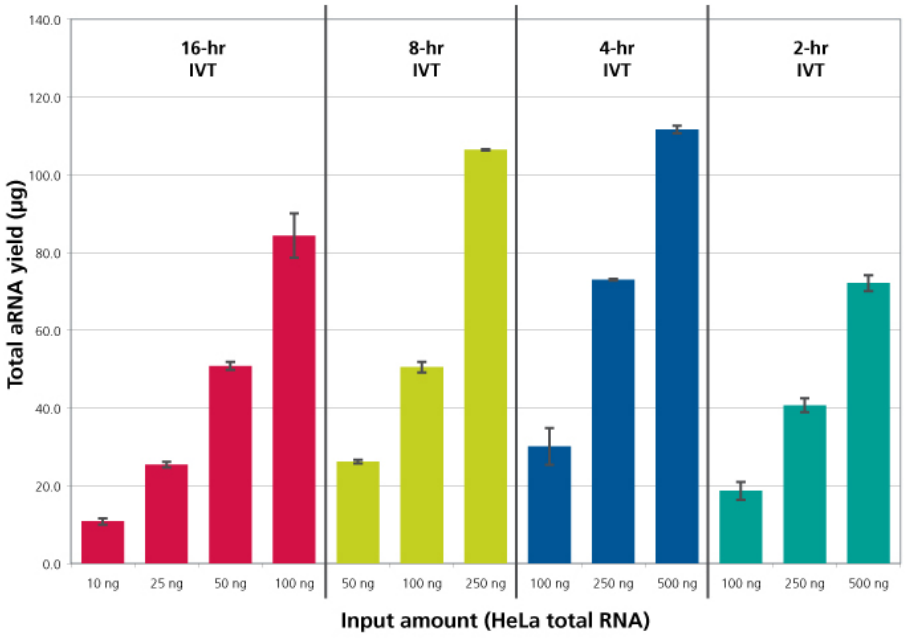


Figure 2: Average aRNA generated by the 3′ IVT Express Kit with different incubation times and input amounts of HeLa total RNA.

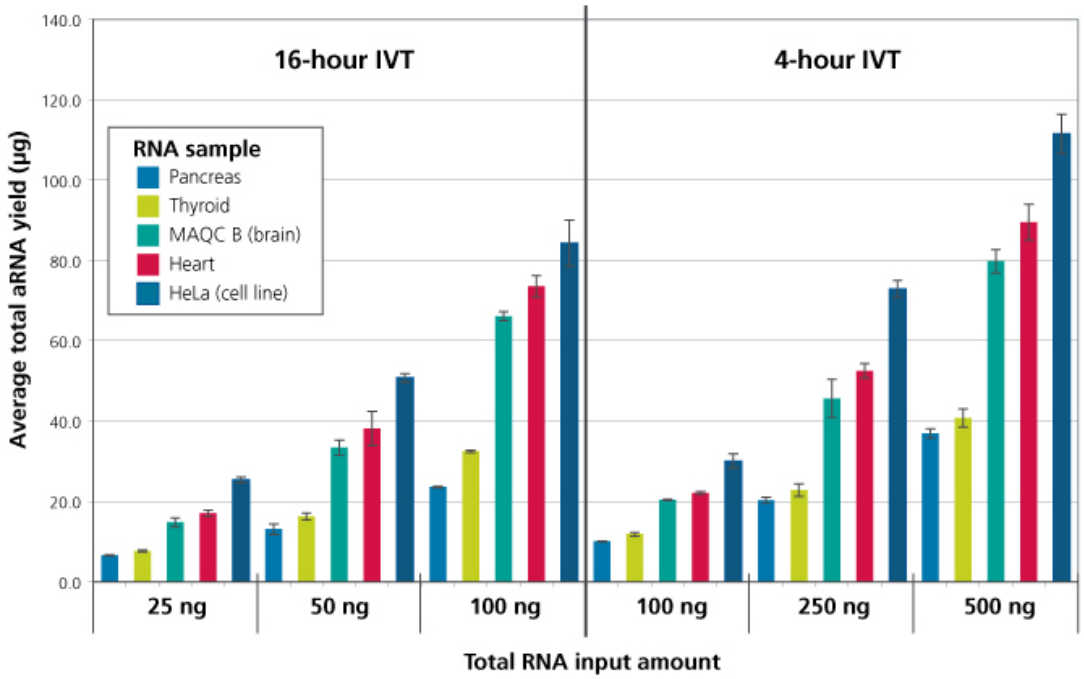


Results

aRNA yield

Figure 2 shows the average aRNA yields calculated for various inputs of HeLa total RNA using 4 different IVT incubation times (2, 4, 8, and 16 hours). Three replicates were processed for each data point; error bars represent one standard deviation. For the largest-format array (49 format), a yield of just over 20 µg of total aRNA (in a 50 µL volume after purification) is required for the fragmentation step. Thus, using HeLa total RNA, the 3′ IVT Express Kit generates sufficient aRNA yields with inputs as low as 25 ng for a 16-hour IVT reaction and as low as 100 ng for a 4-hour IVT reaction.

Figure 3: Average yield of the 3′ IVT Express Kit across a tissue panel with different incubation times and input amounts.



Highly purified RNA from the HeLa cell line may not be representative of typical total RNA samples. Therefore, we further explored the aRNA yields achieved from a variety of total RNA samples (Figure 3). For this experiment, we selected two RNA samples in particular (extracted from human pancreas and human thyroid tissues) that typically produce lower than average aRNA yields. With the 3′ IVT Express Kit, these 2 RNA samples generated sufficient aRNA with inputs as low as 100 ng for a 16-hour IVT reaction, and 250 to 500 ng with a 4-hour IVT reaction.

Comparison of 3′ IVT Express Kit to One-Cycle Reagents

Target was prepared from HeLa, MAQC A (Stratagene Universal Human Reference RNA), and MAQC B (Ambion Human Brain Reference RNA) total RNA samples using both the 3′ IVT Express Kit and the One-Cycle Reagents. For the One-Cycle Reagents, 1 µg of total RNA was used as input with a 16-hour IVT reaction. For the 3′ IVT Express Kit, two input amounts were tested: 50 ng (16-hour IVT) and 500 ng (4-hour IVT). Labeled target was generated using the recommended protocol for each kit and 10 µg of fragmented and labeled aRNA was hybridized to GeneChip® Human Genome U133 Plus 2.0 Arrays.

MAS5 array QC metrics

The aRNA yields and MAS5 array QC metrics are shown in Table 1. The average aRNA yield using the 3′ IVT Express Kit with 50 ng of input was very similar to the average yields using the One-Cycle Reagents with 1 µg of total RNA input. The percent present (%P) values were also comparable between the two kits and the GAPDH 3′/5′ ratios are very consistent; however, the Actin 3′/5′ ratio tends to be higher for the 3′ IVT Express Kit, particularly for lower total RNA input amounts. This may be due to aRNA transcripts that, on average, are slightly shorter. The Actin 3′/5′ ratio tends to be somewhat dependent on the total RNA source and can vary from tissue to tissue. We therefore recommend focusing on the GAPDH 3′/5′ ratio as a QC metric.

Table 1: Comparison of 3' IVT Express Kit and One-Cycle Reagents yield and quality control metrics.

Total RNA	Kit	Input	IVT time	Rep	aRNA yield (µg)	Avg aRNA yield (µg)	SF	%P	Avg %P	Actin 3'-5' ratio	GAPDH 3'-5' ratio
HeLa	IVT Express	50 ng	16 hour	A	67.1	53.2	14.4	41.7	41.6	2.21	1.01
				B	46.0		13.9	42.1		2.39	1.07
				C	46.5		17.7	41.1		3.06	1.14
	IVT Express	500 ng	4 hour	A	99.9	96.1	10.8	42.5	42.7	1.44	0.97
				B	89.8		12.0	43.0		1.43	0.99
				C	98.6		11.3	42.6		3.83	0.96
	One-Cycle	1 µg	16 hour	A	50.7	49.6	7.8	45.2	44.0	1.50	1.10
				B	46.9		10.9	43.5		1.71	1.06
				C	51.3		12.4	43.3		1.68	1.07
MAQC A	IVT Express	50 ng	16 hour	A	61.6	59.2	12.2	51.6	51.3	2.37	1.15
				B	56.6		11.8	52.2		2.75	1.15
				C	59.4		15.8	50.0		2.51	1.12
	IVT Express	500 ng	4 hour	A	106.1	108.2	9.7	52.6	52.9	1.34	1.02
				B	106.0		8.7	53.3		1.31	1.02
				C	112.5		9.1	52.8		1.24	1.01
	One-Cycle	1 µg	16 hour	A	35.6	49.1	12.1	49.2	50.1	1.75	1.14
				B	54.4		12.2	49.7		1.86	1.15
				C	57.3		11.0	51.4		1.83	1.09
MAQC B	IVT Express	50 ng	16 hour	A	47.2	41.0	12.7	49.3	49.5	3.65	1.33
				B	39.3		14.7	49.3		3.89	1.36
				C	36.6		14.2	50.0		4.49	1.45
	IVT Express	500 ng	4 hour	A	83.6	83.2	9.3	52.3	51.7	2.03	1.22
				B	78.5		10.2	51.8		2.14	1.21
				C	87.6		10.9	51.1		2.06	1.19
	One-Cycle	1 µg	16 hour	A	47.8	45.7	12.0	49.3	50.3	2.65	1.49
				B	51.0		12.7	52.0		2.63	1.27
				C	38.1		11.9	49.7		2.49	1.33

To further explore the differences between the 3' IVT Express Kit and One-Cycle Reagents, we generated scatter plots of RMA probe set signal for the MAQC A and B samples. Figure 4 shows the average signal correlation when the two kit types and input amounts are compared. Pearson correlation coefficients for each of the comparisons are also shown. At the probe set level, there is very strong correlation of signal between the two kits.

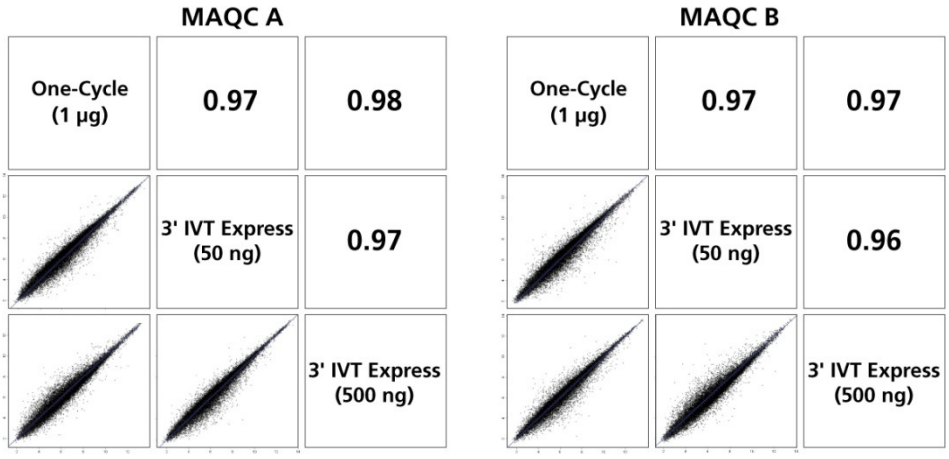


Figure 4: Signal correlation between One-Cycle Reagents and 3' IVT Express Kits with 50 ng (16-hour IVT) and 500 ng (4-hour IVT) total RNA input.

We also calculated the average expression fold change (MAQC A/B) correlation for each of the pair-wise comparisons, as shown in Figure 5. Again, the 3' IVT Express Kit had very high correlation with One-Cycle Reagents at the fold change level.

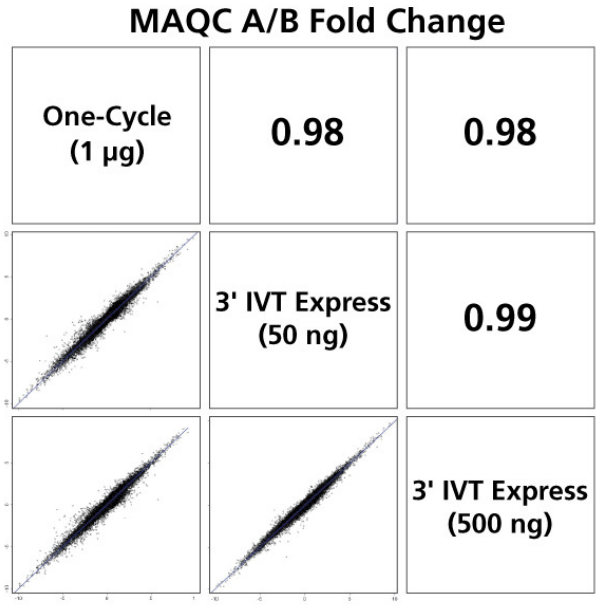


Figure 5: Expression fold change correlation between One-Cycle Reagents and 3' IVT Express Kits with 50 ng (1-hour IVT) and 500 ng (4-hour IVT) total RNA input.

Latin square

To test the sensitivity and specificity of the 3' IVT Express Kit relative to the One- and Two-Cycle Reagents, we carried out a Latin-square experiment. Approximately 20 in vitro transcribed RNA spikes that are not expressed in HeLa cells were distributed into one of four groups and added to HeLa total RNA at four different concentrations (0, 1:200K, 1:100K, 1:50K) to create four pools as shown in Table 2. Target was prepared from three replicates of each pool using either the One-Cycle Reagents, Two-Cycle Reagents, or 3' IVT Express Kits. Sensitivity is tested by measuring how easy it is to distinguish a spike at two concentrations. The probe set signal from two concentrations of the same spiked gene is compared using a t-test.

Table 2: Latin square spike concentrations.

Spike group	Pool 1	Pool 2	Pool 3	Pool 4
Group A	0	1:50,000	1:100,000	1:200,000
Group B	1:200,000	0	1:50,000	1:100,000
Group C	1:100,000	1:200,000	0	1:50,000
Group D	1:50,000	1:100,000	1:200,000	0

Figure 6: Receiver operating characteristic (ROC) plot of the 1:100,000 concentration compared to the 1:200,000 concentration.

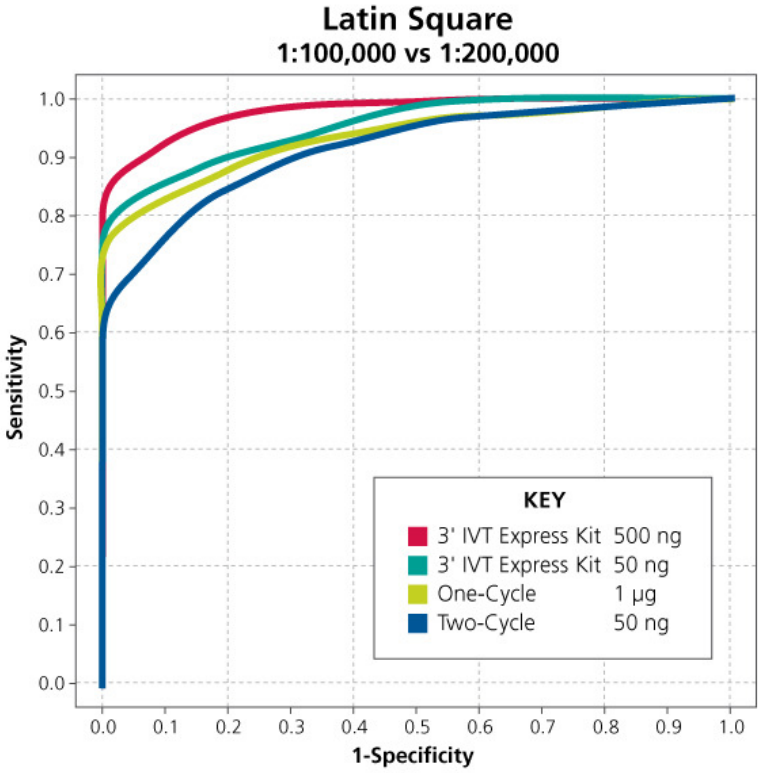


Figure 6 shows a representative ROC plot of the 1:100,000 concentrations compared to the 1:200,000 concentration. The x axis represents sensitivity (true positive) and the y axis represents 1-specificity (false positive). Perfect performance (100 percent true positive, 0 percent false positive) would be shown as a line reaching the upper left corner.

In this experiment, the 3’ IVT Express Kit 500 ng input performs the best. The 3’ IVT Express Kit/50 ng input and One-Cycle Reagents/1 µg input are nearly indistinguishable, and Two-Cycle Reagents demonstrated a slightly lower performance. With this in mind, it is best to standardize an input range and IVT incubation time within an experiment to achieve consistency across experiments.

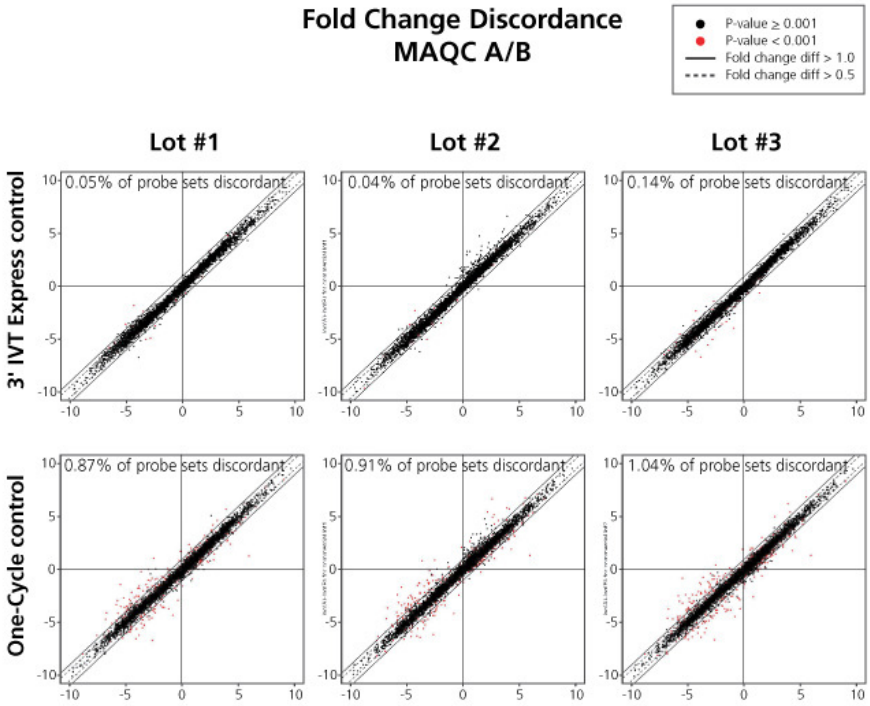


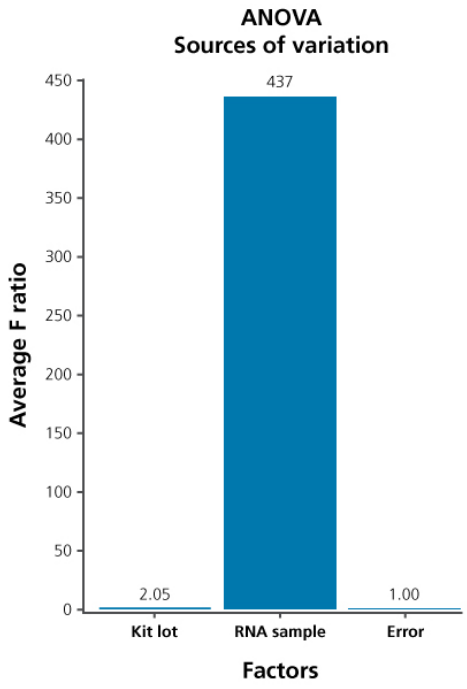
Figure 7: Variation between lots of the 3′ IVT Express Kit and One-Cycle Reagents.

Three-lot validation

Three independent lots of reagents were tested during the development and validation of the 3′ IVT Express Kit. Target was generated from three replicates of the MAQC A and B samples starting with 50 ng of total RNA and using each of the three lots. Labeled target was hybridized to HG-U133 Plus 2.0 Arrays and compared to a control data set that was generated with either a fourth lot of the 3′ IVT Express Kit or One-Cycle Reagents. Figure 7 shows the average fold change (MAQC A/B) of each lot compared to the controls. Probe sets with fold change differences greater than 2.0 and *p*-values less than 0.001 are considered discordant. Each of the three lots of 3′ IVT Express Kit showed nearly identical fold change values (less than 0.15 percent of discordant probe sets).

An analysis of variance (ANOVA) was performed on the data to determine the contribution of variation from the lot of reagents used to generate target. Figure 8 shows the sources of variation for the ANOVA. The contribution of the real biology, as measured by the total RNA sample, was more than 200-fold higher than any variation from the lot of reagents. Variation due to kit lot was close to that of the error term.

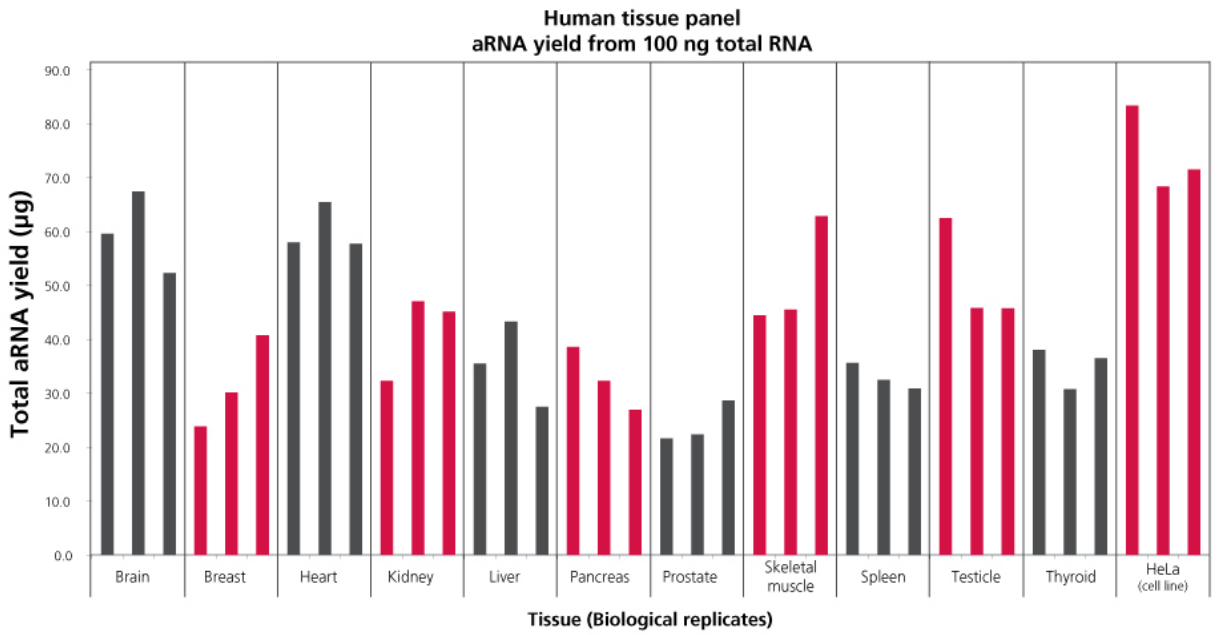
Figure 8: Sources of variation between lots of the 3′ IVT Express Kit.



Tissue panel

Labeled target was generated from biological replicate total RNAs from 11 diverse human tissues and the HeLa cell line. 100 ng of each total RNA sample was used as input into the 3' IVT Express Kit with a 16-hour IVT. Each of the tissues produced varying amounts of total aRNA yield, but each sample resulted in more than 20 µg of aRNA (see Figure 9). 15 µg of each aRNA was fragmented and 10 µg of the fragmented target was hybridized to HG-U133 Plus 2.0 Arrays. The data (CEL files) from this experiment are available online at www.affymetrix.com/products_services/reagents/specific/3_ivtexpresskit.affx.

Figure 9: 3' IVT Express Kit yields across a diverse human tissue panel.



Methods

Target preparation

Labeled aRNA target was generated using the 3' IVT Express Kit according to the instructions in the users' manual (P/N 702646, Rev 1) from three sample sources listed in Table 3. 15 µg of labeled aRNA was fragmented in a 40 µL reaction.

Table 3: Total RNA sample sources.

Total RNA	Description	Vendor	Part No.
HeLa	Cervical Adenocarcinoma (HeLa-S3)	Ambion	7852
MAQC A	Universal Human Reference RNA	Stratagene	740000
MAQC B	Human Brain Reference RNA	Ambion	6050

Hybridization

250 µL of the hybridization cocktail was made with 12.5 µg of fragmented aRNA using the GeneChip® Hybridization, Wash, and Stain Kit (P/N 900720). 200 µL of hybridization cocktail (10 µg of aRNA target) was applied to HG-U133 Plus 2.0 Arrays (P/N 900467) and hybridized for 16

hours at 45°C. Hybridized arrays were washed and stained on the GeneChip® Fluidics Station 450 using reagents in the Hybridization, Wash, and Stain Kit. Washed and stained arrays were scanned using a GeneChip® Scanner 3000.

Data processing
For each experiment, resulting CEL files were quantile normalized in Expression Console™ Software and probe sets were summarized using the RMA algorithm. Percent present (%P) values were generated in Expression Console Software using the MAS5 algorithm.

Analysis
The ANOVA analysis was carried out using Partek® Genomics Suite™.

Discussion

The GeneChip® 3' IVT Express Kit utilizes a highly optimized IVT reaction that dramatically reduces the total RNA input requirements compared to GeneChip® One-Cycle Target Labeling and Control Reagents. In addition, the workflow has been streamlined to reduce the amount of hands-on time. Those using larger input amounts (250 -500 ng, depending on the RNA source and quality) can complete target preparation in a single day. Because there are subtle but measurable differences when using low (50 ng) or high (500 ng) input amounts, it is highly recommended to standardize to a range of input amounts and a single IVT incubation time.

The target generated using the 3' IVT Express Kit produces array results that are very comparable to One-Cycle Reagents, with signal correlations greater than 0.96 and fold change correlation above 0.98. Within the 3' IVT Express Kit, the lot-to-lot variation is also very low, with fewer than 0.15 percent of probe sets showing a fold change discordance. In a Latin-square experiment designed to measure specificity and sensitivity, the 3' IVT Express Kit performed equal to or slightly better than One-Cycle Reagents when discriminating a two-fold difference of spiked-in transcripts at very low concentrations.

Overall, the 3' IVT Express Kit represents a robust and easy-to-use target labeling system that produces results on 3' expression arrays that are very comparable to One-Cycle Reagents.