

GeneChip® Eukaryotic Small Sample Target Labeling Assay Version II*

Introduction

There is an overwhelming and continuing demand for a well characterized protocol that amplifies and labels as low as a few nanograms (ng) of total RNA for expression profiling using GeneChip® probe arrays. This capability is especially important for studies that focus on utilizing a homogenous cell population, where the amount of starting material becomes the limiting factor. This applies to samples from laser capture microdissection, small biopsies, and flow-sorted cells.

Affymetrix published the first version of a research protocol (Version I) in the GeneChip Eukaryotic Small Sample Target Labeling Technical Note in 2001. This Technical Note described reproducible and satisfactory results from 50–100 ng of total RNA. It was based on the principle of performing two cycles of cDNA synthesis and *in vitro* transcription (IVT) reactions for target amplification.

We continued to optimize the assay conditions in ways such as fine tuning the volume of each reaction, amount of primers, as well as incubation time for the IVT reactions.

The Small Sample Labeling Protocol version II (vII) is shown to reproducibly amplify antisense RNA from 10 to 100 ng

*Users who do not purchase the GeneChip T7-Oligo(dT) Promoter Primer Kit may be required to obtain a license under U.S. patent Nos. 5,569,584; 5,716,785; 5,891,636; 6,291,170 and 5,545,522 or to purchase another licensed kit.

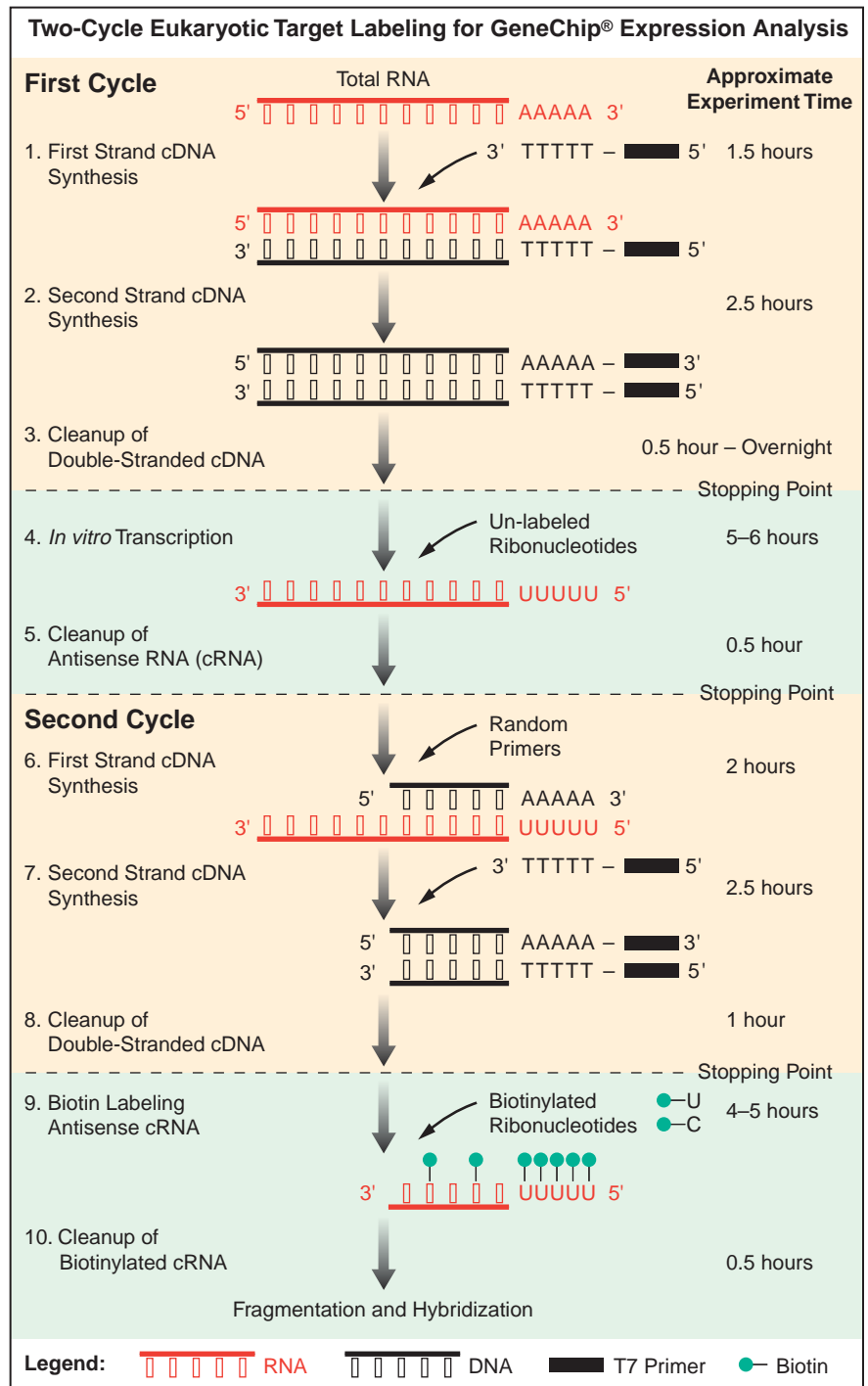


Figure 1. Schematic representation of the Two-Cycle Eukaryotic Target Labeling Assay for GeneChip® Expression Analysis.

of total RNA, or the approximate equivalence to total RNA extracted from 1,000 to 10,000 cells. As a result of the increased optimization, multiple assay performance metrics such as cRNA yield, the 3' to 5' Signal Intensity ratios of control probe sets were improved.

Results

Total RNA was isolated from cultured human HeLa cells. Samples ranging from 1 ng to 100 ng were used in each Small Sample Labeling Protocol vII reaction (described in detail in Appendix II, with the required reagents listed in Appendix I). As a reference, the targets were also prepared following the standard procedure, described in the *GeneChip Expression Analysis Technical Manual*, using 5 µg of the same HeLa total RNA.

Results are presented for the following assay performance criteria, in order to evaluate the effectiveness of the two-cycle amplification for GeneChip array expression analysis.

- **Labeled cRNA yield**
- **Standard array quality metrics including Raw Q, Background, Scaling Factor, Percent Present Calls, and 3' and 5' Signal Intensity ratio of control probe sets**
- **Linearity and sensitivity of amplification as quantified using spike-in bacterial poly-A controls**
- **Replicate analysis and reproducibility**
- **Concordance analysis between the standard and the Small Sample Labeling Protocol vII**

Labeled cRNA Yield

The yield of labeled cRNA is critical because sufficient target needs to be generated for hybridization onto the GeneChip probe arrays. The required amount of labeled cRNA is 10–15 µg for each genome array.

A series of experiments were carried out using different amounts of starting material and the results are summarized in Figure 2. The cRNA targets were prepared by using either the standard assay (5 µg of total RNA) or the Small Sample Labeling Protocol vII (100 ng, 50 ng, 10 ng, and 1 ng of total RNA). The quantity of cRNA obtained was measured by absorbance at 260 nanometers (nm).

The average yields and standard deviation from the six replicates were plotted (Figure 2) to demonstrate the repeatability of amplification reactions. As shown in Figure 2, the quantities of the labeled cRNA obtained from samples of 10–100 ng total RNA with the Small Sample Labeling Protocol vII were comparable with the range anticipated from the standard protocol.

The amount of template became too limiting for the 1 ng samples with only about 10 µg of cRNA targets produced.

Standard Array Quality Metrics

To further evaluate whether the Small Sample Labeling Protocol vII is suitable for preparing targets for GeneChip array expression analysis, 10 µg of cRNA targets, generated from the experiments described previously, were hybridized on the GeneChip Human Genome U133A (HG-U133A) arrays.

The data were then analyzed using Affymetrix' Microarray Suite 5.0 (MAS 5.0) software and various standard array quality control metrics were obtained. The results from the two protocols were evaluated and compared. Each metric is represented by the average value (Avg), as well as the standard deviation (SD) of the six replicates.

We first examined the basic array data such as Raw Q, Background, and Scaling Factor to evaluate the overall sample quality with the targets prepared

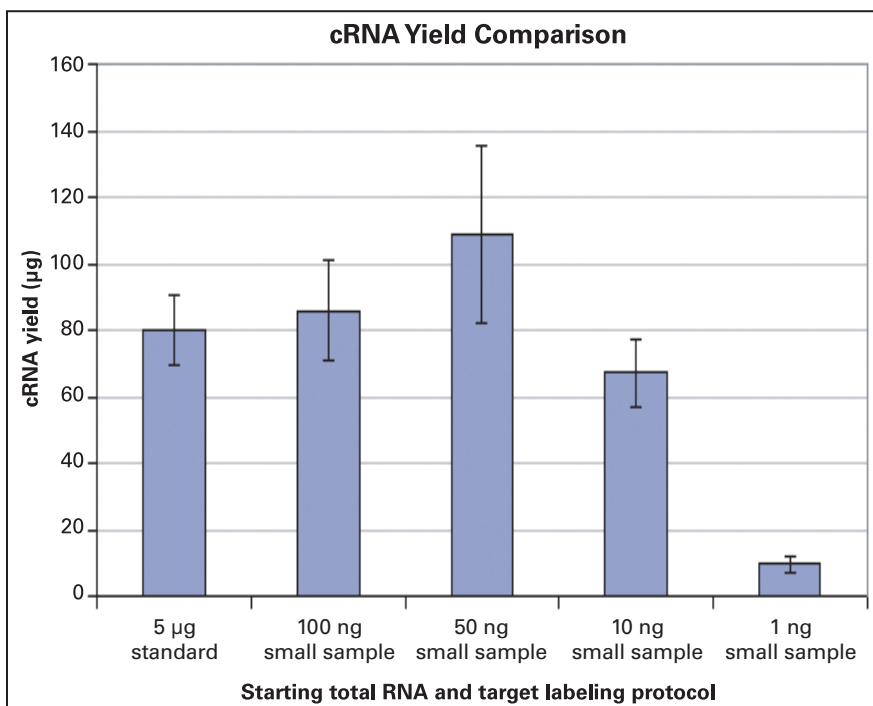


Figure 2. Comparison of cRNA yields. Average and standard deviation from 6 replicates are shown.

according to the two protocols (Version II and the standard protocol).

As shown in Table 1, comparable values were obtained for all three parameters. For example, the Background values were all within the 20–100 range, as anticipated for typical experiments. The Scaling Factors were also within threefold range—even when comparing the data from 1 ng of starting material amplified with the Small Sample Labeling Protocol vII.

The Percent Present Calls comparison was used to globally assess the data representations. As shown in Table 2, comparable Percent Present Call values have been obtained with reducing amounts of starting materials. Even at 1 ng of starting total RNA, about 40 percent of the probe sets were called as Present.

These results demonstrate significant improvement of the Small Sample Labeling Protocol vII, compared with the first version of the protocol, in delivering comparable representations of various transcripts from smaller size samples.

GeneChip arrays are designed with probes selected from the 600 bases adjacent to the poly-A tail. This design strategy along with the inherent generation of shorter fragment from additional amplifications may create targets that are skewed to the 3' end.

To examine this phenomenon, probe sets were created for specific maintenance genes (e.g., GAPDH, beta actin). These probe sets are designed to the 3', middle, and 5' regions of the transcript. The 3' probe set Signal Intensity is then compared to the 5' probe set Signal Intensity (3'/5' ratio) to evaluate the quality of starting material and efficiency of target preparation.

As shown in Table 2, the 10 ng total RNA sample with the Small Sample

Table 1. Array Quality Metrics Comparisons: Raw Q, Background, and Scaling Factor.

Metric	Data	5 µg, Standard	100 ng, Small Sample	50 ng, Small Sample	10 ng, Small Sample	1 ng, Small Sample
Raw Q	Avg	2.25	2.28	2.30	2.13	1.84
	SD	0.21	0.11	0.19	0.05	0.18
Background	Avg	58.66	58.39	58.93	52.92	45.41
	SD	8.04	3.50	6.26	1.76	6.79
Scaling Factor	Avg	1.00	0.80	0.79	1.25	2.53
	SD	0.15	0.14	0.39	0.37	0.65

Table 2. Array Quality Metrics Comparisons: Percent Present Call, 3'/5', and 3'/Middle Ratios.

Metric	Data	5 µg, Standard	100 ng, Small Sample	50 ng, Small Sample	10 ng, Small Sample	1 ng, Small Sample
% Present Call	Avg	48.0%	47.7%	46.4%	44.7%	40.6%
	SD	0.7%	1.3%	1.2%	2.3%	2.9%
GAPDH 3'/5' Ratio	Avg	0.87	2.03	1.78	3.06	3.82
	SD	0.04	0.12	0.22	0.64	0.78
GAPDH 3'/M Ratio	Avg	0.97	1.31	1.15	1.41	1.50
	SD	0.06	0.09	0.06	0.18	0.23
Actin 3'/5' Ratio	Avg	0.96	5.60	9.91	13.09	28.75
	SD	0.03	1.12	2.91	4.46	10.29
Actin 3'/M Ratio	Avg	0.85	2.02	2.71	3.41	5.47
	SD	0.06	0.28	0.59	1.47	1.96

Labeling Protocol vII, produced the 3'/5' ratio for GAPDH within the recommended range of 3, only slightly higher than those samples processed with the standard protocol.

We also calculated the 3'/Middle probe set ratio (3'/M) of the GAPDH gene because the M probes lie approximately 430–770 bases from the most 3' end (Figure 3) and may be a more realistic representation of reliability of the array data. The resulting 3'/M ratios for the GAPDH gene were below 2 for all samples assayed.

Similarly, 3'/5' and 3'/M ratios for a second control gene, beta actin, were

also documented. These ratios deteriorated more rapidly than those for GAPDH consistent with the longer length of the beta actin mRNA (see Figure 3). However, the 3'/M ratio was still 3 on average when using as little as 10 ng of total RNA with the Small Sample Labeling Protocol vII.

Linearity

The ability of any amplification protocol to accurately detect differences in expression levels is highly dependent on the assay's linearity and sensitivity. The Small Sample Labeling Protocol vII was evaluated for both parameters by analyzing the spike-in poly-A control

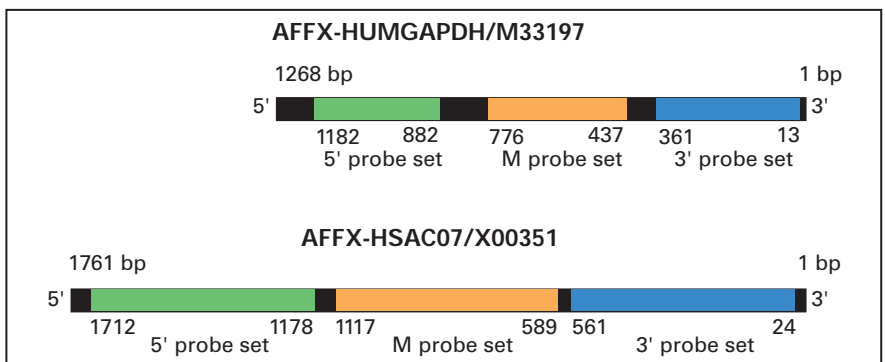


Figure 3. Diagram of the Probe Set Positions for Human GAPDH and Beta Actin. Probe sets are defined by numbered regions starting with one at the 3' end. These numbers do not correlate to the specific nucleotide sequence across the transcript.

transcripts in a complex sample. Four *in vitro*-generated bacterial poly-A controls—*dap*, *lys*, *phe*, and *thr*—were spiked into the complex human HeLa total RNA samples at various concentrations.

After labeling 10 ng of total RNA with the Small Sample Labeling Protocol vII, the target was hybridized on HuGeneFL arrays, and the Signal Intensities for the controls were plotted (Figure 4). The results were comparable to the data generated with the standard procedure (data not shown).

Sensitivity

The sensitivity of the assay was estimated with the lowest concentration of the control transcripts. Specifically, at a 1:81,000- complexity ratio (assuming an average transcript length of 2,000 bases), the controls were present at about three to four copies per cell, in the complex samples. Using 10 ng of total RNA as starting material and amplified with the Small Sample Labeling Protocol vII, all four bacterial control spike-in probe sets were called as Present in all instances. This result indicates that the sensitivity of the assay was maintained in this study even with the additional cycle of amplification.

Reproducibility

Reproducibility is a primary requirement for any amplification protocol, and it is essential for generating reliable results.

Two independent target preparations with 10 ng of total RNA using the Small Sample Labeling Protocol vII were hybridized to HG-U133A arrays. Using the MAS 5.0 software, a scatter plot was generated (Figure 5), illustrating assay reproducibility by comparing the Signal Intensities obtained from the duplicate targets.

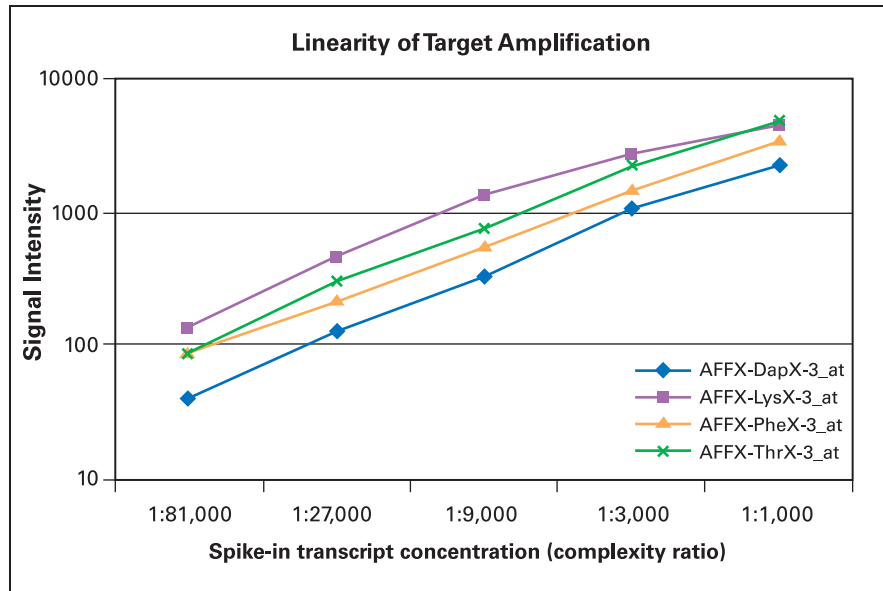


Figure 4. Assessment of Amplification Linearity. Evaluation of transcripts spiked into a complex human HeLa total RNA sample was performed using Microarray Suite 5.0 software.

The obtained r^2 value of 0.98 was consistent with those values observed when comparing results from the two standard labeling procedures (data not shown).

To further assess the reproducibility and degree of assay variability associated with the Small Sample Labeling Protocol vII, the average coefficient of variability (CV) of replicates for individual probe sets was examined.

Comparing the standard labeling procedure (using 5 μ g of total RNA) and the Small Sample Labeling Protocol vII (using 10 ng and 100 ng of the same sample), six independent labeling reactions were analyzed.

As shown in Figure 6, the average CV percent is very comparable with either protocol. These results are indicated by the nearly overlapping trendlines over the entire range of Signal Intensity values. Only probe sets having Signal

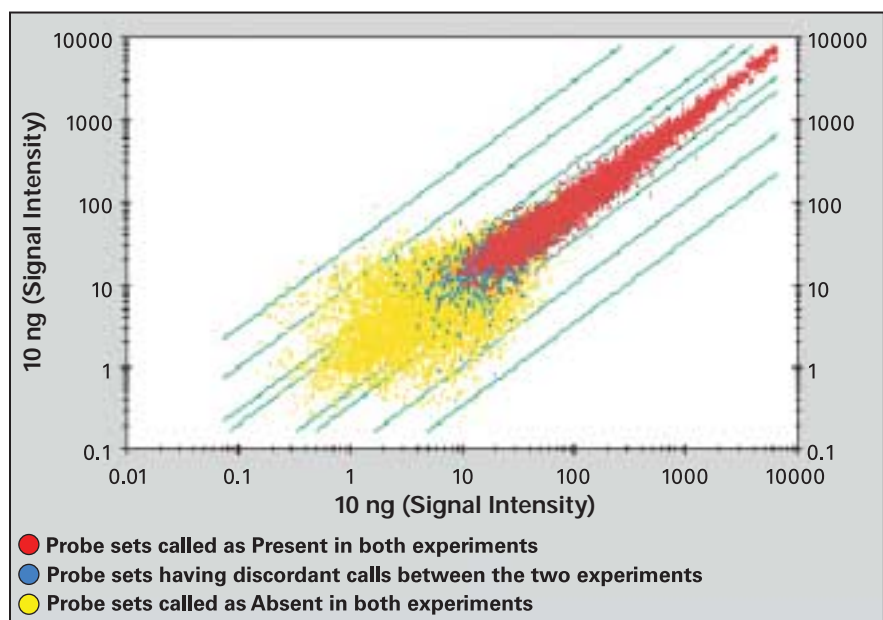


Figure 5. Reproducibility of the Small Sample Labeling Protocol vII. Comparison of Signal Intensities from duplicate samples are shown.

Intensities over 100 are shown in Figure 6 since the majority of the probe sets with Signal Intensities below 100 were called as Absent.

However, when amplifying 10 ng of total RNA with the Small Sample Labeling Protocol vII, the average CV percent increases from 10 percent to approximately 20 percent (Figure 6). We speculate that such an increase is related to the increased difficulty associated with handling a very small amount of material, especially in the first cDNA synthesis steps.

Data Concordance Between the Standard and the Small Sample Labeling Protocol

After hybridizing to HG-U133A arrays, Signal Intensities were compared between the 5 µg samples and the 10 ng samples (Figure 7). The resulting r^2 value of 0.90 indicates that the two protocols were highly similar, but did not produce identical results.

As the standard and Small Sample Labeling Protocol vII use different total RNA amounts and different numbers of amplification cycles, we do not recommend comparing results obtained from these labeling protocols. This was evident from the correlation obtained from the comparison between the standard versus the Small Sample Labeling Protocol vII data (Figure 7).

To further investigate if the additional cycle in the Small Sample Labeling Protocol vII introduced amplification bias, we conducted the detection call concordance analysis.

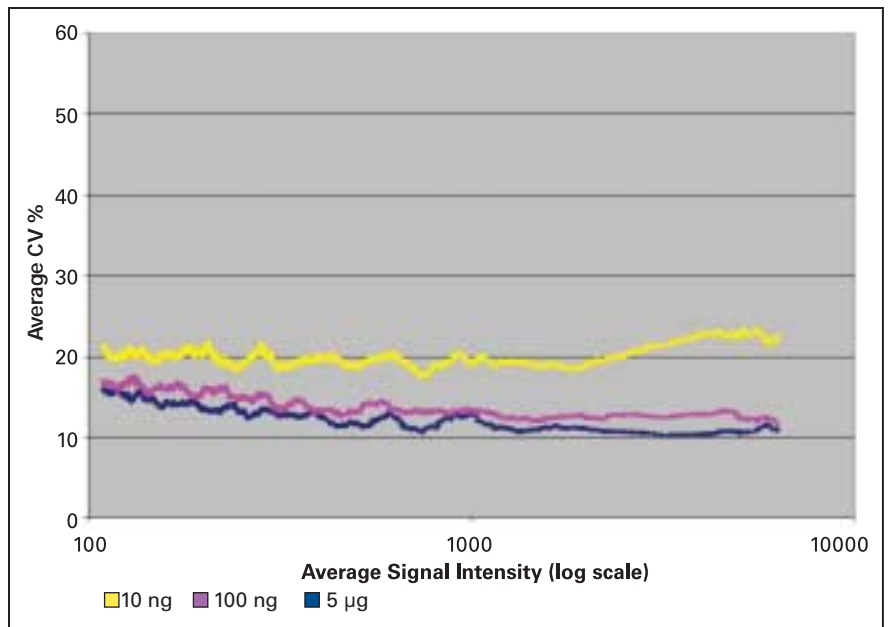


Figure 6. Reproducibility of the Small Sample Labeling Protocol vII. Comparison of average CVs between a 5 µg sample processed with the standard protocol and 10 and 100 ng samples processed with the Small Sample Labeling Protocol vII is shown. The trendlines representing a 200-probe set moving average of the CVs were plotted against the average Signal Intensity.

Using both assays, various amounts of starting materials were labeled, and the data were analyzed using the MAS 5.0 software and its default analysis parameters. The detection calls were compared between samples to calculate the percentage of probe sets that had present/absent calls in common in the two experiments. Conversely, the

percentage of discordant probe sets were identified and compared. The results are summarized in Table 3.

With 10 ng of starting material in duplicate two-cycle labeling experiments, the detection call concordance analysis results provided a baseline as a “best case” scenario (shown in Table 3A).

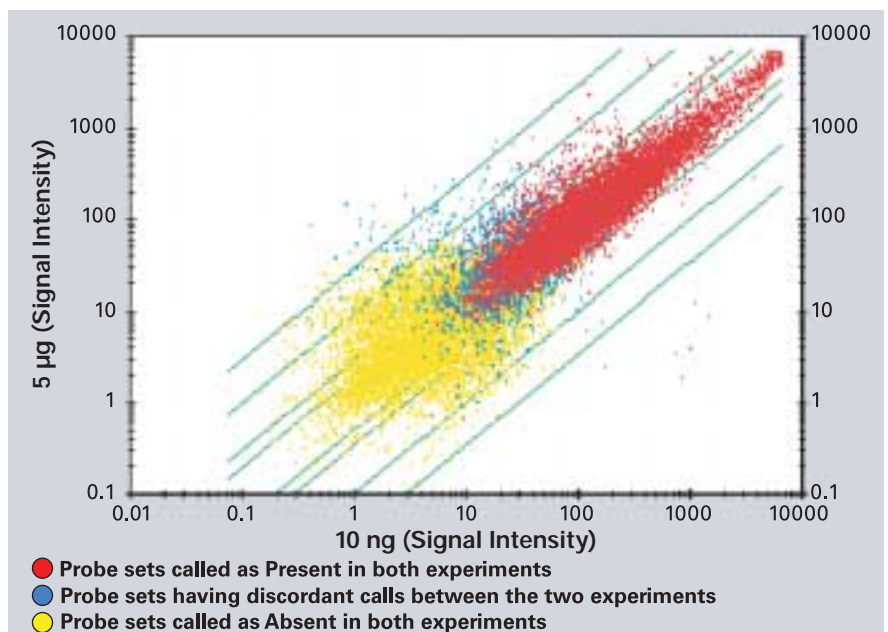


Figure 7. Comparison between the Small Sample Labeling Protocol vII and the Standard Procedure.

Less than 10 percent of the probe sets were categorized as “Discordant” calls, and the breakdown of these calls was similar to those observed when comparing the two standard labeling experiments (data not shown).

Tables 3B, C, D, and E demonstrate similar results, comparing 5 μ g total RNA from the standard assay to gradually reducing amounts of starting materials from 100 ng down to 1 ng with the Small Sample Labeling Protocol vII.

Conclusions

We found that the Small Sample Labeling Protocol vII is suitable for robustly amplifying and labeling as low as 10 ng of total RNA for expression profiling using GeneChip probe arrays.

The assay demonstrated good cRNA yield, sensitivity, reproducibility, and linear amplification.

Table 3. Detection Call Concordance Analysis.

		10 ng	
A.		P	A
10 ng	P	41.4%	4.0%
	A	1.9%	49.6%

		5 μ g	
B.		P	A
100 ng	P	42.7%	4.9%
	A	4.0%	45.2%

		5 μ g	
C.		P	A
50 ng	P	42.2%	4.6%
	A	4.4%	45.5%

		5 μ g	
D.		P	A
10 ng	P	41.7%	3.9%
	A	5.0%	46.3%

		5 μ g	
E.		P	A
1 ng	P	38.7%	2.9%
	A	7.7%	47.5%

Appendix I: Reagents and Materials Used in the Small Sample Labeling Protocol vii

Reagent Name	Vendor	Part Number
DEPC-treated water, 4L	Ambion	9920
T7-Oligo(dT) Promoter Primer, 50 μ M, HPLC-purified 5' – GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG – (dT) ₂₄ – 3'	Affymetrix	900375
SuperScript II, 200 U/ μ L, 40,000U 5X first strand buffer and 0.1M DTT included	Invitrogen	18064-071
dNTP mix, 10 mM, 100 μ L	Invitrogen	18427-013
RNase inhibitor, 40 U/ μ L, 10,000U	Ambion	2684
5X second strand buffer, 500 μ L	Invitrogen	10812-014
DNA ligase, <i>E. coli</i> , 10 U/ μ L, 100U	Invitrogen	18052-019
DNA polymerase I, <i>E. coli</i> , 10 U/ μ L, 1,000U	Invitrogen	18010-025
RNase H, 2 U/ μ L, 120U	Invitrogen	18021-071
T4 DNA polymerase, 5 U/ μ L, 250U	Invitrogen	18005-025
Glycogen, 5 mg/mL, 5,000 μ L	Ambion	9510
Ammonium Acetate (NH ₄ OAc), 5M	Ambion	9070G
MEGAscript T7 Kit	Ambion	1334
RNeasy Mini Kit	QIAGEN®	74104
β -Mercaptoethanol	Sigma	M-3148
Random primers, 3 μ g/ μ L	Invitrogen	48190-011
ENZO® BioArray™ HighYield™ RNA Transcript Labeling Kit, 10 reactions	Affymetrix	900182
Miscellaneous Reagents		
Ethanol, absolute, kept cold		
Ethanol, 70%, kept cold		

Appendix II: Small Sample Labeling Protocol vII

Recommendation: It is important to familiarize yourself with the protocol and handling of small amounts of samples by practicing the procedure first with diluted total RNA samples.

Important points to note before starting:

1. Make a fresh dilution of the T7-Oligo(dT) promoter primer (5 μ M), the random primers (0.2 μ g/ μ L), and total RNA (1 ng–100 ng, if diluted total RNA is used) each time.
2. When adding reagents, mix the components thoroughly by gently pipetting up and down several times. Do not vortex the sample.
3. To ensure consistent results, keep the incubation time as close as possible to the instructions within the protocol.

First Cycle of Amplification

Step 1. First cycle, first strand cDNA synthesis

Note: Use a thermal cycler with a heated lid for incubations in this step. The following program can be used as a reference; the 4°C hold steps are inserted to allow for reagent additions.

70°C	6 minutes
4°C	hold
42°C	60 minutes
70°C	10 minutes
4°C	hold

- a. Mix the total RNA sample and the T7-Oligo(dT) promoter primer thoroughly in a 0.2 mL PCR tube:

Total RNA, 1 ng–100 ng	1 μ L
T7-Oligo(dT) promoter primer, 5 μ M	1 μ L
Total volume	2 μL

- b. Incubate the sample at 70°C in a thermal cycler for 6 minutes.
c. Cool the sample to 4°C for 2 minutes.
d. Spin the tube briefly to collect the sample at bottom of tube.
e. Prepare the RT_Premix_1 as follows. It is recommended to assemble the master mix for at least four reactions to avoid pipetting very small volumes.

	1 rxn	4 rxns	___ rxns
DEPC-treated water	0.375 μ L	1.5 μ L	
5X first strand buffer	1 μ L	4 μ L	
DTT, 0.1M	0.5 μ L	2 μ L	
dNTP mix, 10 mM	0.375 μ L	1.5 μ L	
RNase inhibitor, 40 U/ μ L	0.25 μ L	1 μ L	
SuperScript II, 200 U/ μ L	0.5 μ L	2 μ L	
Total volume	3 μL	12 μL	

- f. Add 3 μ L of the RT_Premix_1 to the denatured RNA and primer mixture, making a final volume of 5 μ L.
g. Mix the components thoroughly by pipetting gently up and down several times.
h. Spin the tube briefly to collect the sample at bottom of tube.
i. Incubate the sample at 42°C for 1 hour.
j. Heat the sample at 70°C for 10 minutes to inactivate SuperScript II.
k. Spin the tube briefly and cool the sample to 4°C.

Step 2. First cycle, second strand cDNA synthesis

Note: Use a thermal cycler without a heated lid for incubations in this step. The following program can be used as a reference; the 4°C hold steps are inserted to allow for reagent additions.

16°C	120 minutes
4°C	hold
16°C	10 minutes
4°C	hold

- a. Prepare the SS_Premix_1 as follows. It is recommended to assemble the master mix for at least four reactions to avoid pipetting very small volumes.

	1 rxn	4 rxns	__ rxns
DEPC-treated water	22.75 μ L	91 μ L	
5X second strand buffer	7.5 μ L	30 μ L	
dNTP mix, 10 mM	0.75 μ L	3 μ L	
DNA ligase, <i>E. coli</i> , 10 U/ μ L	0.25 μ L	1 μ L	
DNA polymerase I, <i>E. coli</i> , 10 U/ μ L	1 μ L	4 μ L	
RNase H, 2 U/ μ L	0.25 μ L	1 μ L	
Total volume	32.5 μL	130 μL	

- b. Add 32.5 μ L of the SS_Premix_1 to each first strand reaction, making a final volume of 37.5 μ L.
 c. Mix the components thoroughly by gently pipetting up and down a few times.
 d. Spin the tube briefly to collect the sample at bottom of tube.
 e. Incubate the sample at 16°C for 2 hours.
 f. Add 1 μ L of T4 DNA polymerase (5 U/ μ L) to the reaction.
 g. Mix the components thoroughly by gently pipetting up and down a few times.
 h. Continue to incubate the sample at 16°C for 10 minutes.

Step 3. First cycle, double-stranded cDNA cleanup by ethanol precipitation

- a. Transfer the reaction, from the previous step, to a 1.5 mL centrifuge tube.
 b. Add 80 μ L of DEPC-treated water to dilute the reaction.
 c. Add 2 μ L of glycogen (5 mg/mL), 0.6 volumes (72 μ L) of 5M NH₄OAc, and 2.5 volumes (480 μ L) of cold absolute ethanol.
 d. Mix the components well by pipetting up and down several times.
 e. Precipitate the double-stranded cDNA at -20°C. For 10–100 ng of starting total RNA, the precipitation time may be 30 minutes to 2 hours. For 1–10 ng of starting total RNA, overnight precipitation usually generates more consistent results.
 f. Centrifuge the tube at 14,000 rpm for 20 minutes at 4°C.
 g. Remove the supernatant carefully and wash the pellet by adding 800 μ L of 70% cold ethanol.
 h. Centrifuge the tube at 14,000 rpm for 5 minutes at 4°C.
 i. Remove the ethanol carefully.
 j. Dry the pellet in a vacuum centrifuge for approximately 5–10 minutes.

Step 4. First cycle, IVT for cRNA amplification using Ambion MEGAscript T7 Kit

- Notes**
- Prepare premixed NTPs by adding equal amounts of each of the four NTPs (75 mM) provided in the kit.
 - To ensure that the cDNA sample is dissolved completely, it is important to mix the reaction very well after adding water and NTP mix to the dried cDNA pellet.
 - The minimum IVT incubation time is 5 hours. This is particularly important if the starting sample amount is as low as 1 ng of total RNA or from less than 100 cells.
 - To bring down the condensation formed during the IVT incubation, it is important to mix the reaction by gently tapping and briefly spinning the tube every 30–45 minutes.
 - In order to avoid sample loss, it is not recommended to transfer the sample to a fresh tube at this stage. Perform the incubation in the 1.5 mL centrifuge tube in a water bath.

- a. At room temperature, add the following reagents to the dried double-stranded cDNA pellet, in the order indicated:

	1 rxn	__ rxns
DEPC-treated water	4 μ L	
Premixed NTPs, 18.75 mM each	4 μ L	
10X reaction buffer	1 μ L	
10X enzyme mix	1 μ L	
Total volume	10 μL	

- b. Mix the components thoroughly after adding each reagent by gently pipetting up and down a few times.
- c. Spin the tube briefly to collect the sample at bottom of tube.
- d. Incubate the sample at 37°C in a water bath for 5–6 hours.

Step 5. First cycle, cRNA cleanup with RNeasy columns

- a. Add 90 µL of RNase-free water to the cRNA product from the previous step.
- b. Follow the RNeasy Mini Protocol for RNA Cleanup in the handbook from QIAGEN that accompanies the RNeasy Mini Kit for cRNA purification.
- c. In the last step of purification, elute the cRNA sample with 30 µL of RNase-free water first, and follow with an additional 20 µL of water for the second elution.
- d. To determine cRNA yield, remove 2 µL of the cRNA, and add to 98 µL of water to measure the absorbance at 260 nm.
- e. Use the following table to calculate the volume of cRNA to be used in the second cycle amplification and labeling reactions:

Starting Total RNA	cRNA Yield After First IVT	cRNA to be Used for Second Cycle
100 ng	1–2 µg in 50 µL	≥10 µL or (200–400 ng)
50 ng	1–2 µg in 50 µL	≥10 µL or (200–400 ng)
10 ng	Undetectable	All
1 ng	Undetectable	All

Note: If the yield is less than those indicated in the table, use the entire RNeasy column eluate (50 µL) for the second cycle of cDNA synthesis.

- f. Transfer the appropriate volume of cRNA from above to a fresh 1.5 mL centrifuge tube and use vacuum centrifuge with medium heat to reduce the sample volume to no more than 4 µL. Avoid complete drying of the liquid. The cRNA can be stored at -20°C at this point before proceeding to the next step.

Note: If the cRNA sample is completely dried in the vacuum centrifuge, add 10 µL of RNase-free water and incubate at 42°C for 10 minutes to resuspend the sample completely. The volume will then need to be reduced to no more than 4 µL in the vacuum centrifuge before proceeding to the next step.

Second Cycle of Amplification and Labeling

Step 6. Second cycle, first strand cDNA synthesis

Notes: Use heat blocks for incubations in this step.

- a. Add random primers to the cRNA sample in the 1.5 mL centrifuge tube and mix thoroughly:

cRNA, variable	4 µL
Random primers, 0.2 µg/µL	1 µL
Total volume	5 µL

- b. Spin the tube briefly to collect sample at bottom of tube.
- c. Incubate the sample at 70°C for 10 minutes.
- d. Cool the sample on ice for 2 minutes.
- e. Prepare the RT_Premix_2 as follows. It is recommended to assemble the master mix for at least two reactions to avoid pipetting very small volumes.

	1 rxn	2 rxns	___ rxns
5X first strand buffer	2 µL	4 µL	
DTT, 0.1M	1 µL	2 µL	
dNTP mix, 10 mM	0.5 µL	1 µL	
RNase inhibitor, 40 U/µL	0.5 µL	1 µL	
SuperScript II, 200 U/µL	1 µL	2 µL	
Total volume	5 µL	10 µL	

- f. Add 5 μL of the RT_Premix_2 to the denatured RNA and primer mixture, making a final volume of 10 μL .
- g. Mix the components thoroughly by gently pipetting up and down several times.
- h. Spin the tube briefly to collect sample at bottom of tube.
- i. Incubate the sample at 42°C for 1 hour and spin briefly.
- j. To remove the RNA template, add 1 μL of RNase H (2 U/ μL) and incubate the sample for 20 minutes at 37°C.
- k. Heat the sample at 95°C for 5 minutes to inactivate RNase H.
- l. Chill the sample on ice for 2 minutes.
- m. Spin the tube briefly to collect sample at bottom of tube.

Step 7. Second cycle, second strand cDNA synthesis with T7-Oligo(dT) promoter primer

Note: Use heat blocks and a water bath for incubations in this step. Alternatively, transfer sample to a 0.2 mL PCR tube and perform the incubations in a thermal cycler without a heated lid. The following program can be used as a reference; the 4°C hold steps are inserted to allow for reagent additions.

70°C	6 minutes
4°C	hold
16°C	120 minutes
4°C	hold
16°C	10 minutes
4°C	hold

- a. Add 2.0 μL of the 5 μM T7-Oligo(dT) promoter primer to the chilled sample from the previous step.
- b. Mix the sample thoroughly by gently pipetting up and down a few times.
- c. Spin the tube briefly to collect sample at bottom of tube.
- d. Incubate the sample at 70°C for 6 minutes.
- e. Cool the sample to 4°C and spin the tube briefly.
- f. Prepare the SS_Premix_2 as follows:

	1 rxn	___ rxns
DEPC-treated water	43.5 μL	
5X second strand buffer	15 μL	
dNTP mix, 10 mM	1.5 μL	
DNA polymerase I, <i>E. coli</i> , 10 U/ μL	2 μL	
Total volume	62 μL	

- g. Add 62 μL of the SS_Premix_2 to the sample, making a total volume of 75 μL .
- h. Mix the components thoroughly by pipetting gently up and down a few times.
- i. Spin the tube briefly to collect sample at bottom of tube.
- j. Incubate the sample at 16°C for 2 hours.
- k. Add 2 μL of T4 DNA polymerase (5 U/ μL) to the reaction and mix thoroughly.
- l. Incubate the sample at 16°C for 10 minutes.

Step 8. Second cycle, double-strand cDNA cleanup by ethanol precipitation

- a. To the reaction tube, add 2 μL of 5 mg/mL glycogen, 0.6 volume of 5M NH_4OAc , and 2.5 volumes of cold absolute ethanol.
- b. Mix the reaction well by pipetting up and down several times.
- c. Precipitate the double-stranded cDNA at -20°C for 30 minutes.
- d. Centrifuge the tube at 14,000 rpm for 20 minutes at 4°C.
- e. Remove the supernatant carefully and wash the cDNA pellet by adding 800 μL of 70% cold ethanol.
- f. Centrifuge the tube at 14,000 rpm for 5 minutes at 4°C.
- g. Remove the ethanol carefully.
- h. Dry the sample in a vacuum centrifuge for approximately 5–10 minutes, or until dry. The cDNA pellet can be stored at -20°C overnight before proceeding to Step 9.

Step 9. Second cycle, IVT for cRNA amplification and labeling with ENZO BioArray HighYield RNA Transcript Labeling Kit

Notes Use a water bath for incubations in this step.

- a. At room temperature, add the following reagents to the dried double-stranded cDNA pellet, from the previous step, in the following order:

	1 rxn	___ rxns
DEPC-treated water	22 μ L	
10X HY reaction buffer	4 μ L	
10X Biotin labeled ribonucleotides	4 μ L	
10X DTT	4 μ L	
10X RNase inhibitor mix	4 μ L	
20X T7 RNA polymerase	2 μ L	
Total volume	40 μL	

- b. Mix the components thoroughly after adding each reagent, especially after adding water, ensuring the cDNA pellet is completely dissolved.
- c. Spin the tube briefly to collect sample at bottom of tube.
- d. Incubate the sample at 37°C for 4–5 hours. To bring down condensation formed during incubation, mix the sample reaction by gently tapping and briefly spinning the tube every 30–45 minutes.

Step 10. Second cycle, labeled cRNA target cleanup with RNeasy columns

- a. Add 60 μ L of RNase-free water to the cRNA product from the previous step.
- b. Follow the RNeasy Mini Protocol for RNA Cleanup in the handbook from QIAGEN that accompanies the RNeasy Mini Kit for cRNA purification.
- c. In the last step of cRNA purification, elute the product with 30 μ L of RNase-free water first, and then follow with another 20 μ L of water for the second time.
- d. Remove 2 μ L of the labeled cRNA, and add it to 98 μ L of water in order to measure the absorbance at 260 nm, determining the cRNA yield.

Step 11. Fragmentation and Hybridization

Follow the instructions described in the *GeneChip® Expression Analysis Technical Manual* for the fragmentation, hybridization, washing, and staining steps.

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