

Microarray Results, *Pronto*

The Pronto!TM *Plus* System: Increasing Microarray Reproducibility, Reliability and Ease of Use

By Michael W. Briggs, Ph.D., Corning Life Sciences, and Nadine Nassif, M.S., Promega Corporation



Abstract

The Pronto!TM Plus Systems, developed jointly by Corning Incorporated and Promega Corporation, are fully integrated systems of qualified microarray components. The systems provide reagents and protocols specifically optimized for microarray applications, including components for RNA isolation, cDNA labeling, cDNA clean-up, solutions for printing and hybridization, as well as Corning[®] UltraGAPSTM slides. The quality and consistency of these integrated systems ensure inter- and intra-lab consistency and reproducibility when generating microarray data. In addition, these integrated systems provide the convenience of obtaining all critical microarray components from a single source.

Pronto!TM Plus Systems provide an optimized, integrated set of reagents and protocols that give users a versatile, beginning-to-end solution for microarray analysis.

Introduction

DNA microarray analysis is a powerful tool, giving biologists the potential to monitor an entire transcriptome and assess the expression of thousands of genes simultaneously. To generate high-quality, interpretable data, great care must be taken in several distinct but interrelated procedures.

Many current microarray protocols require combining reagents from multiple suppliers or require users to prepare and validate “home brew” reagents, making standardization a challenge. However, Pronto!TM *Plus* Systems^(a) provide an optimized, integrated set of reagents and protocols that give users a versatile, beginning-to-end solution for microarray analysis. By providing all required components (with the exception of the Cy[®]-labeled nucleotides) together in an optimized, standardized package, the Pronto!TM *Plus* Systems enable self-printing users to achieve highly reproducible and robust array results.

In the experiments described here, human cDNA microarrays printed onto UltraGAPSTM slides were used to assess parameters of target labeling and microarray hybridization. Labeled cDNA was generated from 5µg of total RNA template using the ChipShotTM Labeling and Clean-Up components^(b) of the Pronto!TM *Plus* Systems. Target cDNAs were labeled by direct incorporation of Cy[®] dye-labeled nucleotides. Results demonstrate that the Pronto!TM *Plus* Systems and protocols provide a framework for achieving superior, reproducible signal detection for more reliable data interpretation during a microarray experiment.

SV Total RNA Isolation

The SV Total RNA Isolation System^(c,d) component of the Pronto!TM *Plus* Systems is an affinity purification method for consistent isolation of high-quality total RNA (Figure 1). The cells are lysed in a chaotropic buffer, and the RNA is precipitated and captured on a silica matrix. A DNase I treatment step eliminates genomic DNA from the sample (1). Purification is achieved without the use of phenol/chloroform extractions or ethanol precipitations, and there is no DNase carryover in the final RNA preparation (1). The isolation system provides a fast and simple technique for the preparation of purified and intact total RNA from tissues, cultured cells and white blood cells. While yield will vary depending on starting material (Table 1), a single SV Total RNA System isolation routinely provides sufficient RNA for multiple cDNA labeling reactions.

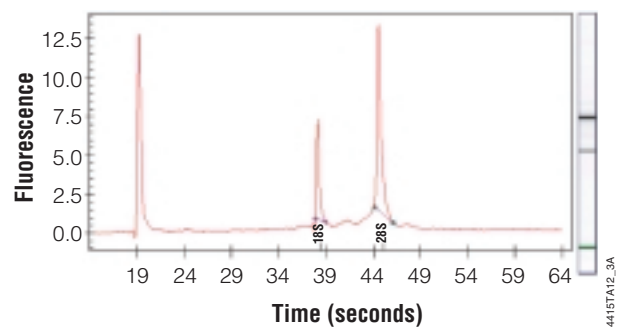


Figure 1. The quality and purity of total RNA isolated from HeLa cells using the SV Total RNA Isolation System is demonstrated on an Agilent 2100 Bioanalyzer. The ratio of 28S to 18S eukaryotic ribosomal RNA is consistently observed to be within the range of 1.5 to 2.5 when using the SV Total RNA Isolation System, indicating RNA integrity suitable for molecular biology analysis (2). Total RNA with a ratio of [28S / 18S] = 2.13 was isolated from 2×10^6 HeLa cells as described in the Pronto!TM *Plus* Systems Technical Manual #TM243.

Table 1. Average Yields of Total RNA Isolated From Cells and Tissue Using the SV Total RNA Isolation System.

Samples	Amount Processed	Avg. Yield per Prep (µg)
Mouse Tissues		
Liver	30mg	131
Kidney	20mg	44
Spleen	15mg	79
Muscle	30mg	22
Brain	60mg	39
Rat Tissues		
Pancreas	30mg	100
Heart	60mg	16
Lung	60mg	36
Cell Line		
RAW264.7	5 × 10 ⁶	51
HeLa	2 × 10 ⁶	35
293T	2 × 10 ⁶	15

ChipShot™ Labeling and Labeling Clean-Up

The ChipShot™ Labeling System allows users to generate fluorescently labeled cDNA via direct incorporation of Cy[®]-labeled nucleotides in a reverse transcription reaction. The ChipShot™ System is capable of generating labeled cDNA from minimal amounts of either total RNA or mRNA template. When using total RNA only 5µg of template is required, compared to many other commercially available systems that require 10–25µg of template. When using mRNA, only 1.5µg of template is required. Labeling cDNAs derived from mRNA or total RNA involves different dNTP mixes and different protocols optimized for each specific type of template.

Moreover, the ChipShot™ System protocols have been optimized to account for differences when incorporating Cy[®]3- and Cy[®]5-labeled nucleotides. Cy[®]5 tends to be incorporated less efficiently than Cy[®]3, therefore a greater volume of unlabeled dNTPs is included in the Cy[®]5 reaction to promote cDNA production.

The ChipShot™ Labeling Clean-Up System efficiently removes unincorporated nucleotides while allowing excellent recovery of dye-labeled cDNA to keep backgrounds low and signals high during hybridization. The system also incorporates an RNase treatment for removal of the RNA template following cDNA synthesis, which allows accurate quantitation of cDNA yield.

Assessing the quality and quantity of the labeled cDNA is essential for generating reproducible and interpretable hybridization data. Following a cDNA labeling reaction and clean-up, absorbance readings (260, 550 and 650nm) should be taken using undiluted cDNA. Diluting the cDNA prior to reading the absorbance may give inaccurate readings due to low concentrations that may be outside the linear range of detection of some

spectrophotometers. These absorbance readings are used to calculate the cDNA yield (ng), the pmol of dye incorporated (Cy[®]3 or Cy[®]5) and the frequency of incorporation (FOI). FOI is defined as the number of Cy[®]-labeled nucleotides incorporated per 1,000 nucleotides of cDNA.

$$\text{Amount of labeled cDNA (ng)} = A_{260} \times 37 \times \text{total volume (}\mu\text{l)}$$

$$\text{For Cy}^{\text{®}}\text{3: pmol of dye incorporated} = (A_{550} \times \text{total volume (}\mu\text{l)})/0.15$$

$$\text{For Cy}^{\text{®}}\text{5: pmol of dye incorporated} = (A_{650} \times \text{total volume (}\mu\text{l)})/0.25$$

$$\text{FOI} = (\text{pmol of dye incorporated} \times 324.5)/\text{ng of cDNA}$$

These equations were generated using the following constants: Average Molar Mass of dNTP = 324.5; One A₂₆₀ unit of single-stranded DNA = 37µg/ml; Extinction Coefficient of Cy[®]3 = 150,000M⁻¹cm⁻¹ at 550nm; Extinction Coefficient of Cy[®]5 = 250,000M⁻¹cm⁻¹ at 650nm.

Best results from microarray hybridizations are obtained with cDNAs that fall into the ranges given in Table 2.

Table 2. Expected Results for cDNA Yield and FOI.

5µg Total RNA	Yield cDNA (ng)	Dye Incorporated (pmol)	FOI
Cy [®] 3	1,200–2,400	100–170	20–35
Cy [®] 5	900–2,400	45–120	12–25
1.5µg mRNA			
Cy [®] 3	350–650	40–75	25–45
Cy [®] 5	325–650	20–50	15–35

The ChipShot™ Labeling and Labeling Clean-Up Systems are optimized to yield efficient synthesis of cDNA while maintaining a frequency of incorporation recommended for expression profiling experiments. Sufficient labeled cDNA is consistently generated for hybridization to 2–3 full 22 × 60mm arrays (Table 3).

Hybridization

UltraGAPS™ slides are coated with an extremely even layer of Gamma Amino Propyl Silane. The GAPS surface binds spotted DNA via an ionic interaction. Heat or UV treatment is used to crosslink the DNA to the surface for covalent attachment. Smaller DNA molecules and oligonucleotides are best immobilized by UV crosslinking. These procedures work equally well for DNA molecules longer than 300bp. When baking, take care regarding the cleanliness of the oven. Volatile organics can irreversibly contaminate the surface of the array, leading to high backgrounds.

Depending on their age, the purity of the biological material and other reagents used, and the storage conditions, DNA microarrays may develop significant levels of background fluorescence on and around the printed areas. It is important to eliminate such background autofluorescence in order to accurately measure the levels of transcript abundance.

The Pronto![™] Universal Pre-Soak treatment followed

Pronto!™ Plus Systems for Microarray Analysis...continued

Table 3. Experimental Results for cDNA Yield and FOI.

Sample	Yield cDNA (ng)	Dye Incorporated (pmol)	FOI
Lung Total RNA (commercial prep) n = 7			
Cy [®] 3	1,582 ± 60	155 ± 7	32 ± 1
Cy [®] 5	1,735 ± 98	83 ± 6	16 ± 1
HeLa Total RNA (SV Total RNA Isolation System) n = 8			
Cy [®] 3	1,519 ± 82	156 ± 10	33 ± 1
Cy [®] 5	1,628 ± 149	106 ± 13	21 ± 1
Lung mRNA (commercial prep) n = 8			
Cy [®] 3	527 ± 22	68 ± 5	42 ± 2
Cy [®] 5	515 ± 64	40 ± 7	25 ± 2
293T mRNA (PolyAtract[®] mRNA Isolation System) n = 8			
Cy [®] 3	446 ± 22	57 ± 5	42 ± 2
Cy [®] 5	452 ± 18	32 ± 2	23 ± 1

Total RNA from a commercial source and total RNA isolated with the SV Total RNA Isolation System (Cat.# Z3100) were used as templates for cDNA labeling. Yields and FOI were consistent within and between sample types. Similarly, mRNA from a commercial source and mRNA isolated using the PolyAtract[®] mRNA Isolation System (Cat.# Z5210) were also used as templates for synthesis of labeled cDNA. Again, yields and FOI were consistent and reproducible. All experimental results fall within the range of expected results from Table 2. Total RNA was isolated from 2 x 10⁶ HeLa and from 2 x 10⁶ 293T cultured cells using the SV Total RNA Isolation System as described in the *Pronto!™ Plus Systems Technical Manual #TM243*. Messenger RNA was isolated from HeLa and 293T cultured cells using the RNAgents[®] Total RNA Isolation System (Cat.# Z5110) in combination with the PolyAtract[®] mRNA Isolation System as described in the Technical Manuals #TB087 and #TM021, respectively. Labeled cDNA was generated from total RNA or mRNA using the ChipShot™ Labeling and Labeling Clean Up Systems from 5µg total RNA template or 1.5µg of mRNA template, as described in the *Pronto!™ Plus Systems Technical Manual #TM243*.

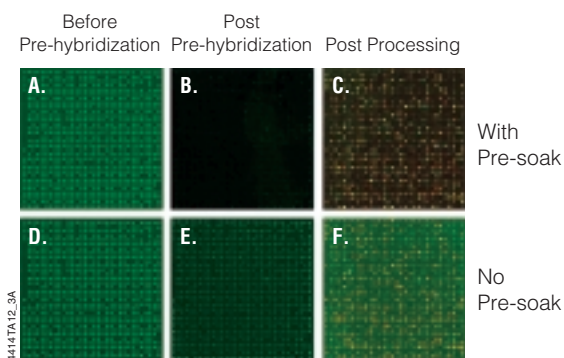


Figure 2. Pre-soak treatment reduces Cy[®]3 autofluorescence, resulting in low background and improved discrimination. Arrays were printed on UltraGAPS™ slides using the Pronto!™ Spotting Solutions, as described in the *Pronto!™ Plus System Technical Manual #TM243*. All printing was performed at Corning[®] Incorporated. Slides were scanned using identical settings using an Axon GenePix[®] 4000B microarray scanner and analyzed using GenePix[®] software before any array processing (left panels), after prehybridization (middle panels), or after two-color hybridization (right panels). Arrays were printed and stored longer than 2 years, and significant autofluorescence has accumulated on the printed content of the slides (left panels). Slides were prehybridized identically with the exception that the top slide was also incubated with Pre-Soak Solution to remove contaminating autofluorescence (middle panels). The Pre-Soak treatment dramatically reduced background and increased the number of detectable features (right panels).

by Pre-Hybridization is efficient at eliminating background fluorescence (3), thus effectively yielding a broader dynamic range for data generation (Figure 2).

The surface of the UltraGAPS™ slides is highly reactive toward DNA. The key to producing high-quality microarrays is to take advantage of this high reactivity during the printing process while minimizing the spurious attachment of nucleic acids to the unprinted area during subsequent manipulation of the array. The Pronto!™ Pre-Hybridization and Hybridization solutions contain sufficient protein and nucleic acid blockers to result in highly specific hybridization signals with extremely low backgrounds.

Because the UltraGAPS™ slides are designed for use with long oligos and cDNA, the hybridization solution is of higher stringency so that optimal results are obtained with an overnight hybridization at 42°C. Moreover, the Pronto!™ Universal hybridization solution contains enhancers that increase the kinetics of DNA duplex formation, ensuring more robust expression data. The use of a Corning[®] Hybridization Chamber (Corning[®] Cat. #2551) allows the flexibility of hybridization in a waterbath, hybridization oven or incubator at the user's convenience.

The combination of the Pronto!™ Plus reagents and protocols with the UltraGAPS™ slides yields highly reproducible data both within and between experiments (Figure 3). The graph shown in Figure 3, Panel B, depicts the %CV for three independent labeling and hybridization experiments, each of which consists of 3–4 slides. Raw data were normalized in each channel against the mean of the total signal across the arrays, and the Cy[®]3: Cy[®]5 ratios were calculated for more than 3,000 genes per array (blank spots and negative controls were removed prior to analysis). Results of these analysis consistently show less than 10% variation between slides, providing a greater degree of confidence when assessing the validity and interpretability of microarray data.

Conclusions

The Pronto!™ Plus Systems provide start-to-finish solutions for microarray experimentation, enabling self-printing microarrays to achieve highly reproducible and robust array results. By integrating the components together in an optimized package, the Pronto!™ Plus Systems not only provide convenience to the user but also increase data quality and lower failure rate, thus reducing repeated experiments.

The ChipShot™ cDNA labeling chemistry is optimized for the use of 5µg total RNA template, as opposed to the 10–25µg required by many other commercially available

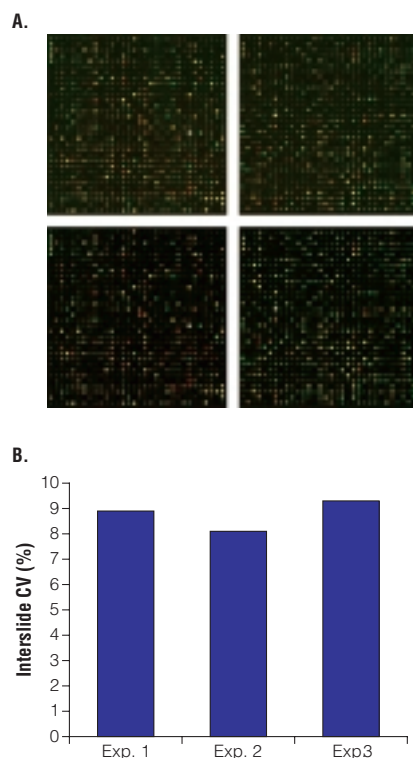


Figure 3. Experimental reproducibility when using the Pronto!™ Plus Systems. **Panel A.** Subgrids from four representative 4K human cancer arrays (provided by Corning® Incorporated) are shown. Labeled cDNA was generated with the ChipShot™ components of the Pronto!™ Plus System using 5µg of total RNA template. RNAs were from cultured HeLa cells (Cy®3) or 293T cells (Cy®5) isolated with the SV Total RNA Isolation System components. **Panel B.** Reproducibility was assessed by determining the coefficient of variation (%CV = Std. Dev./Mean) for 4,000 features from n = 3–4 arrays, in three independent experiments (including independent cDNA labelings). Interslide %CV for each experiment was below 10%, indicating a high degree of reproducibility both within and between experiments. Slides were printed as described in Figure 2. Hybridization was performed using the Pronto!™ Universal Reagent System, as described in the *Pronto!™ Plus Systems Technical Manual #TM243*. Arrays were scanned using an Axon GenePix® 4000B microarray scanner and analyzed using the Axon GenePix® software.

systems. With 5µg of total RNA, one labeling reaction yields sufficient fluorescent cDNA for multiple full arrays. The requirement for less template allows users to conserve limited samples and/or increase the number of technical replicates performed.

A high degree of reproducibility is observed both within and between experiments when using the Pronto!™ Plus Systems, with interslide %CV shown to be less than 10%. In addition to reproducibility, the Pronto!™ Systems chemistry is optimized for high sensitivity and low background (Figure 2); sensitivity studies have shown that as little as 3pg in 5µg total RNA can be detected (1 copy/cell in 5×10^5 tissue culture cells (4); data not shown). This level of sensitivity enables users to discover changes in low-expressing genes.

In less than a decade the microarray field has evolved to the point where standardization should be a prerequisite for implementation. The Minimum Information About a Microarray Experiment (MIAME) guidelines are an excellent first step toward that end. The MIAME guidelines ensure that microarray data can be easily interpreted and independently verified (5). Optimized protocols such as those in the Pronto!™ Plus Systems that are all-encompassing of the microarray process provide direction toward the goal of simplified MIAME reporting, manuscript submission, and improved ability to work with collaborators.

Acknowledgments

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Additional Information

- ◆ Pronto!™ Systems Web Site (www.prontosystems.com)

Protocols

- ◆ *Pronto!™ Plus Systems Technical Manual #TM243*, Promega Corporation. (www.promega.com/tbs/tm243/tm243.html)

Ordering Information

Product	Size	Cat.#
Pronto!™ Plus System 1 ^(a)	10 reactions	40051
Pronto!™ Plus System 2 ^(a)	10 reactions	40052
Pronto!™ Plus System 3 ^(a)	10 reactions	40053
Pronto!™ Plus System 4 ^(a)	10 reactions	40054
Pronto!™ Plus System 5 ^(a)	25 reactions	40055
Pronto!™ Plus System 6 ^(a)	25 reactions	40056

SV Total RNA Isolation System ^(c,d)	50 preps	Z3100
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^(a)U.S. Pat. Nos. 6,027,945 and 6,368,800, Australian Pat. No. 732756 and other patents and patents pending.

^(b)Certain applications of this product are covered by patents issued and applicable in certain countries. Because purchase of this product does not include a license to perform any patented application, users of this product may be required to obtain a patent license depending upon the particular application and country in which the product is used.

^(c)Australian Pat. No. 730718 and other patents and patents pending.

^(d)U.S. Pat. No. 6,218,531, Australian Pat. No. 745185 and other patents pending.

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