



# Optimal Microarrays, Pronto

## Pronto!<sup>TM</sup> Plus Systems: New Integrated Reagent Systems for Optimal Microarray Performance

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### Abstract

Promega Corporation and Corning Incorporated have developed two new systems that provide fully integrated sets of reagents for use in microarray analysis. The Pronto!<sup>TM</sup> Plus Direct System provides reagents for generating fluorescent cDNA by direct incorporation of Cy<sup>®</sup>-labeled dCTP. The Pronto!<sup>TM</sup> Plus Indirect System allows users to generate fluorescent cDNA via synthesis of aminoallyl-modified cDNA, followed by post-synthesis conjugation of CyDye<sup>TM</sup> NHS ester. Both systems provide reagents and protocols specifically optimized for microarray applications and include components for cDNA labeling and cDNA clean-up as well as solutions for array conditioning and hybridization. Users can also choose versions of both systems that include the SV Total RNA Isolation System, which provides a fast and simple procedure for purifying intact total RNA from a variety of sources. These integrated sets of reagents enable researchers to achieve the highest possible quality, consistency, and reproducibility throughout their microarray processes. Here we describe the system components and present typical data generated using these systems.

**The low RNA requirement of the Pronto!<sup>TM</sup> Plus Systems for generating labeled cDNA provides a significant advantage over other commercially available labeling systems.**

### Introduction

The use of DNA microarrays for transcriptional profiling remains the main application of this powerful technology. Genome-wide monitoring of gene transcription has become a standard tool in research laboratories. Biologists increasingly use DNA microarrays to understand processes as diverse as the mechanisms of toxicity, embryogenesis, and disease progression. The quality and consistency of the reagents and protocols used to process these arrays has a substantial impact on the reliability of the data generated.

The Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems<sup>(a-d)</sup> provide optimized, integrated sets of reagents and protocols that give users start-to-finish solutions for microarray processing. By providing all required components (with the exception of Cy<sup>®</sup>-labeled nucleotides or CyDye<sup>TM</sup> NHS ester) together in an

integrated package, these systems help users achieve the highest possible performance from their microarray experiments while also providing the convenience of a single standardized source for critical reagents.

### Isolate High-Quality RNA for Labeling

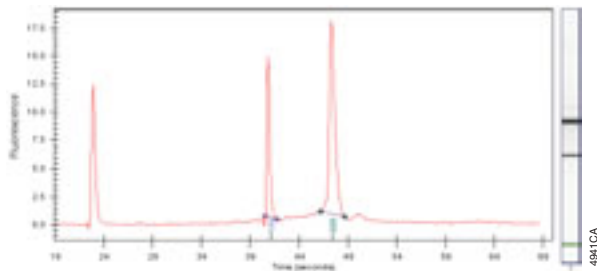
Versions of both the Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems are available that include the SV Total RNA Isolation System<sup>(b,c)</sup>. In the SV Total RNA Isolation System protocols, cells are lysed in a chaotropic buffer, and the RNA is captured and purified on a silica matrix. Contaminating genomic DNA is removed by nuclease treatment. This affinity purification method eliminates the need for phenol/chloroform extractions and is compatible with a wide variety of sample types, including mammalian tissue, cultured cells, white blood cells, plant tissue, bacteria, and yeast (1). A single SV Total RNA Isolation procedure routinely provides sufficient total RNA for multiple cDNA labeling reactions (1).

The SV Total RNA Isolation System typically yields more than 5 $\mu$ g of pure RNA from 10<sup>6</sup> cultured mammalian cells. Depending on cell type, yields can be as high as 17 $\mu$ g per 10<sup>6</sup> cells (2). Figure 1 shows the high purity and integrity of total RNA obtained from 293T cells. The ratio of 28S to 18S of the RNA extracted from 293T cells was 1.97, which is well within the range from 1.5 to 2.5 that is consistently observed for eukaryotic ribosomal RNA. The quantity and quality of the resulting RNA were perfectly suitable for molecular biology experimentation (3).

### Choose the Labeling Method: Direct or Indirect

Fluorescent cDNA labeling can be accomplished by either direct labeling or indirect labeling methods. The Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems provide users with their choice of methods for generating fluorescent cDNA. Both labeling systems are optimized for use of either total RNA or poly(A)<sup>+</sup> mRNA as templates for cDNA synthesis, usually requiring only 5 $\mu$ g of total RNA or 1.5 $\mu$ g of mRNA to generate sufficient labeled cDNA for hybridization to at least two full-size arrays. The low RNA requirement is a significant advantage over other commercially available labeling systems (some of which require as much as 25 $\mu$ g of total RNA template), since it allows users to work with limited amounts of RNA template and increase the number of replicates that can be performed.

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



**Figure 1. Purity and integrity of total RNA isolated using the SV Total RNA Isolation System.** Total RNA was isolated from  $2 \times 10^6$  293T cultured cells as described in the *Pronto!™ Plus Direct and Pronto!™ Plus Indirect Systems Technical Manuals* (#TM243 and #TM261, respectively). A small aliquot was run on the Agilent Bioanalyzer.

The ChipShot™ Direct Labeling System<sup>(d)</sup>, a component of the Pronto!™ Plus Direct System, provides an efficient, reproducible method for generating fluorescent cDNA by direct incorporation of Cy<sup>®</sup>-labeled dCTP during reverse transcription. The ChipShot™ Direct Labeling System protocol is optimized to minimize the effect of differences in the efficiency of incorporating Cy<sup>®</sup>3- versus Cy<sup>®</sup>5-labeled dCTP by reverse transcriptase. Typical yields of direct Cy<sup>®</sup>-labeled cDNA from 5µg total RNA and 1.5µg of mRNA can be as much as 2µg and 400ng, respectively (Table 1). The Cy<sup>®</sup>-labeled cDNA obtained by direct incorporation from total RNA typically contains more than 20 Cy<sup>®</sup>-labeled dCTPs per 1,000 nucleotides. These levels of yield and the frequency of incorporation (FOI) ensure that at least two full-size arrays can be hybridized with Cy<sup>®</sup>-labeled cDNA resulting from a single pair of reactions.

Alternatively, the ChipShot™ Indirect Labeling System, a component of the Pronto!™ Plus Indirect System, provides reagents and protocols for generating fluorescent cDNA without the use of Cy<sup>®</sup>-labeled nucleotides. Indirect labeling is achieved by incorporating aminoallyl-modified nucleotides during cDNA synthesis, followed by conjugation of a CyDye™ NHS-ester dye to the aminoallyl-modified cDNA after the reverse transcription reaction is complete. The higher affinity of reverse transcriptase for aminoallyl-dUTP compared to Cy<sup>®</sup>-labeled dCTP increases cDNA yield to an average of 3.0 and 1.3µg from total RNA and mRNA, respectively. Subsequent conjugation of the dye ester produces Cy<sup>®</sup>3- and Cy<sup>®</sup>5-labeled cDNA having similar FOI, in sufficient quantities for hybridization to three or more full-size arrays (Table 1).

## Recover Pure Labeled cDNA Efficiently

The new ChipShot™ Membrane Clean-Up System is included as the cDNA clean-up component of both the Pronto!™ Plus Direct and the Pronto!™ Plus Indirect Systems. The ChipShot™ Membrane Clean-Up System uses a silica membrane spin column for affinity

purification of the cDNA. This method effectively removes unincorporated nucleotides, primers and free dye while yielding highly efficient cDNA recovery. An RNase treatment step is incorporated after labeling and prior to clean-up; this RNase treatment removes the RNA template following cDNA synthesis, allowing accurate quantitation of the labeled cDNA.

Compared to previous clean-up methods that relied on silica beads or particles for purification, the new ChipShot™ Membrane Clean-Up System provides a faster, simpler technique for efficient recovery of purified cDNA. Used in combination, the ChipShot™ Labeling Systems and the ChipShot™ Membrane Clean-Up System provide superior methods for efficiently synthesizing labeled, high-purity cDNA for use in microarray experiments.

## Process Microarrays with Consistent Results

The Pronto!™ Universal Hybridization Kit is a component of all Pronto!™ Plus Systems. This kit provides all the necessary reagents to prepare arrays, conduct hybridization and wash the arrays following hybridization.

In preparation for hybridization to the Cy<sup>®</sup>-labeled cDNA, arrays must be conditioned by incubation in background reduction reagents (pre-soaking) and pre-hybridization buffer (pre-hybridization). These steps prepare the arrays for hybridization by eliminating “spotted” and substrate autofluorescence and preventing the non-specific attachment of labeled target to the array surface. Minimizing background fluorescence increases the specificity and sensitivity of the arrays and leads to more reliable and reproducible data.

The Pronto!™ Plus Systems now include a new liquid formulation of the active component of the pre-soaking treatment. The new liquid sodium borohydride is equally as effective as the solid form previously used and has the added benefit of allowing the use of exact volumes to match the number of arrays being processed and the capacity of the containers used.

## Obtain High-Quality, Reproducible Microarray Data

To test the relative performance of the labeling systems at the hybridization level, arrays of 32,000 human cDNAs spotted on UltraGAPS™ Coated Slides were hybridized with either direct- or indirect-labeled cDNA. The target cDNA was synthesized from mRNA extracted from two human cell lines, 293T and HeLa, known to display significantly different phenotypes.

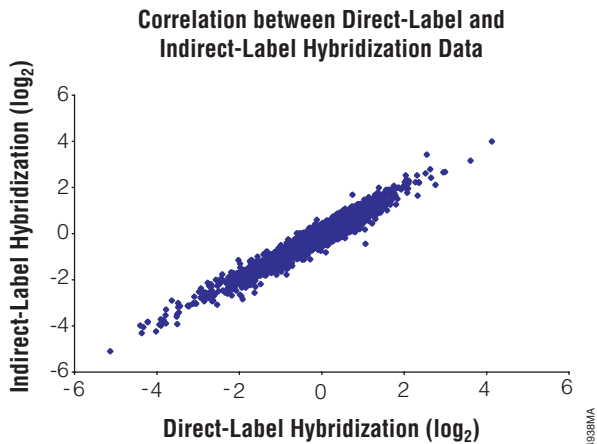
As expected, a relatively large proportion of the genes represented on the array showed differential expression. Of 8,114 spots that passed all filters, 388 spots were found to represent differentially expressed genes (at least twofold either way) by indirect labeling; 90% (350 spots)

**Table 1. Yield and Label Strength of Cy<sup>®</sup>3- and Cy<sup>®</sup>5-Labeled cDNAs Synthesized Using the ChipShot<sup>™</sup> Direct Labeling and Indirect Labeling Systems.**

Source	Yield (ng)	Dye Incorporated (pmol)	FOI
<b>Direct Labeling of cDNA from Total RNA</b>			
<b>Total RNA (commercial source #1)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 32)	1,979 ± 157	194 ± 30	32 ± 4
Cy <sup>®</sup> 5-labeled cDNA (n = 29)	2,138 ± 388	130 ± 33	19 ± 2
<b>Total RNA (commercial source #2)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 24)	2,000 ± 290	195 ± 41	31 ± 3
Cy <sup>®</sup> 5-labeled cDNA (n = 22)	2,200 ± 175	135 ± 24	20 ± 3
<b>293T Total RNA (prepared using SV Total RNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	1,590 ± 39	165 ± 4	34 ± 0
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	1,890 ± 46	115 ± 3	21 ± 0
<b>HeLa Total RNA (prepared using SV Total RNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	1,927 ± 39	232 ± 6	39 ± 0
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	2,166 ± 41	150 ± 2	22 ± 0
<b>Direct Labeling of cDNA from mRNA</b>			
<b>mRNA (commercial source #1)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 22)	473 ± 74	78 ± 16	53 ± 5
Cy <sup>®</sup> 5-labeled cDNA (n = 16)	518 ± 77	59 ± 13	37 ± 3
<b>293T mRNA (prepared using PolyATtract<sup>®</sup> mRNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	350 ± 30	60 ± 5	56 ± 0
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	412 ± 8	46 ± 1	36 ± 0
<b>HeLa mRNA (prepared using PolyATtract<sup>®</sup> mRNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	413 ± 11	70 ± 2	55 ± 0
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	422 ± 12	48 ± 1	37 ± 2
<b>Indirect Labeling of cDNA from Total RNA</b>			
<b>Total RNA (commercial source #1)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 11)	3,151 ± 166	199 ± 30	21 ± 4
Cy <sup>®</sup> 5-labeled cDNA (n = 23)	2,979 ± 355	234 ± 42	25 ± 3
<b>Total RNA (commercial source #2)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 30)	3,100 ± 531	173 ± 40	18 ± 3
Cy <sup>®</sup> 5-labeled cDNA (n = 19)	3,072 ± 477	215 ± 45	23 ± 5
<b>293T Total RNA (prepared using SV Total RNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	2,256 ± 114	164 ± 13	24 ± 1
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	2,957 ± 123	207 ± 45	29 ± 0
<b>HeLa Total RNA (prepared using SV Total RNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	2,913 ± 114	195 ± 4	22 ± 1
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	2,957 ± 123	250 ± 6	27 ± 1
<b>Indirect Labeling of cDNA from mRNA</b>			
<b>mRNA (commercial source #1)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	1,382 ± 236	80 ± 13	19 ± 1
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	1,521 ± 236	111 ± 18	24 ± 1
<b>293T mRNA (prepared using PolyATtract<sup>®</sup> mRNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	1,322 ± 106	92 ± 6	23 ± 2
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	1,215 ± 93	63 ± 9	17 ± 1
<b>HeLa mRNA (prepared using PolyATtract<sup>®</sup> mRNA Isolation System)</b>			
Cy <sup>®</sup> 3-labeled cDNA (n = 3)	1,331 ± 92	95 ± 3	23 ± 1
Cy <sup>®</sup> 5-labeled cDNA (n = 3)	1,386 ± 121	89 ± 18	21 ± 3

Yield and label strength of Cy<sup>®</sup>3- and Cy<sup>®</sup>5-labeled cDNA synthesized using the ChipShot<sup>™</sup> Direct Labeling and the ChipShot<sup>™</sup> Indirect Labeling Systems. Total RNA and mRNA samples used as template for these cDNA-synthesis reactions were either obtained from commercial sources or isolated from human cell lines using the SV Total RNA Isolation System (as described in the *Pronto!<sup>™</sup> Plus Direct and Pronto!<sup>™</sup> Plus Indirect Systems Technical Manuals #TM243 and #TM261, respectively*) or the RNAgents<sup>®</sup> Total RNA Isolation System in combination with the PolyATtract<sup>®</sup> mRNA Isolation System (as described in Technical Manuals #TB087 and #TM021, respectively). Yield and FOI (number of Cy<sup>®</sup>-labeled dCTPs incorporated per 1,000 nucleotides of cDNA) were calculated with the "Labeled cDNA Calculator" found on [www.prontosystems.com](http://www.prontosystems.com) using absorbance values at 260, 550, and 650nm. The number of replicate reactions per sample is shown in parentheses; values include standard deviation.

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



**Figure 2. Correlation between hybridization results obtained from Cy<sup>5</sup>-cDNA synthesized using the ChipShot™ Direct Labeling and the ChipShot™ Indirect Labeling Systems.** Arrays of 32,000 human cDNAs were spotted on UltraGAPS™ Coated Slides at The Institute for Genomic Research. Data filters were applied to eliminate spots that were statistically indistinguishable from background, deviated from the expected size by more than 20%, and showed internal CVs greater than 100%. Additionally, spots for which triplicate data was not available across both conditions after applying these filters were removed from further analysis.

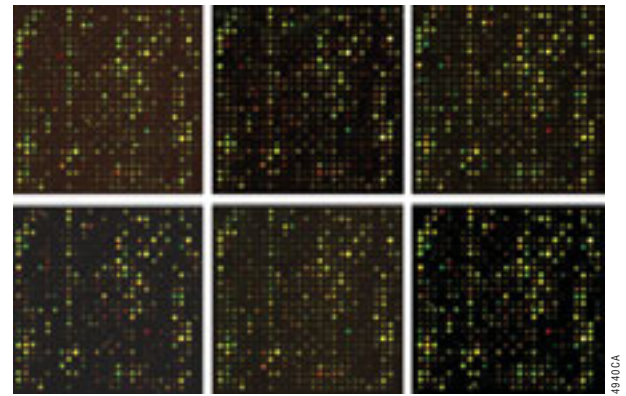
of these presumptive regulatory events were also detected by direct labeling. Overall correlation between the two data sets was 94% (Figure 2). The differences detected in relative fluorescence were all statistically significant ( $p < 0.01$ ), as determined by paired t-test.

Reproducibility across the 12 arrays was equal among HeLa- and 293T-derived samples (Figure 3). Data sets from arrays hybridized with direct- and indirect-labeled cDNAs had coefficients of variation (CV) of 19.3 and 19.5%, respectively. These values are particularly striking given that the HeLa and 293T cDNAs were both hybridized in a reversed-labeled pattern.

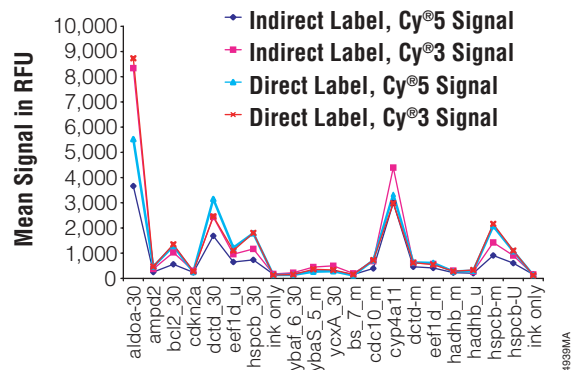
HeLa and 293T cDNAs synthesized from total RNA were also hybridized to a set of 12 high-density arrays. The results of these hybridizations were of similarly high sensitivity and reproducibility. The correlation between ratiometric values obtained from direct- and indirect-labeled cDNA made from total RNA was 95.4%.

The Pronto!™ Plus Systems now also include the new Hybridization Buffer for Short Oligos in addition to the Hybridization Buffer for Long Oligos and cDNAs. The new buffer is recommended for hybridization to arrays of 30- to 50mers.

Twenty-one 30mer oligonucleotides, fifteen of which represented human genes, were spotted onto ten Corning Epoxide Coated Slides. The resulting arrays were divided into two groups of five and separately hybridized using



**Figure 3. Reproducibility of hybridization pattern obtained with Cy<sup>3</sup>- and Cy<sup>5</sup>-labeled cDNA synthesized using the ChipShot™ Indirect Labeling System.** Three 32,000-human-cDNA arrays were hybridized with target Cy<sup>3</sup>-labeled cDNA made from 293T cells and Cy<sup>5</sup>-labeled cDNA made from HeLa cells by indirect labeling (top row); another three arrays were hybridized with target Cy<sup>3</sup>-labeled cDNA made from HeLa cells and Cy<sup>5</sup>-labeled cDNA made from 293T cells (bottom row). Arrays were scanned with an Axon 4000B scanner set at 100% power for both channels and PMT of 700 for Cy<sup>3</sup> and 800 for Cy<sup>5</sup>.



**Figure 4. Correlation between hybridization patterns from Cy<sup>5</sup>-labeled cDNA synthesized using the ChipShot™ Direct and Indirect Labeling Systems on covalently bound short oligonucleotides.** Twenty-one 30mer unmodified oligonucleotides, fifteen of which represented human genes, were purchased from commercial vendors and spotted onto Corning Epoxide Coated Slides. The arrays were hybridized with target cDNA dissolved in Pronto!™ Short-Oligo Hybridization Buffer. Arrays were scanned with an Axon 4000B scanner set at 100% power for both channels and PMT of 700 for Cy<sup>3</sup> and 800 for Cy<sup>5</sup>.

direct- and indirect-labeled cDNAs. Average signal and background intensities obtained from the two array groups were similar, the latter not exceeding 150 relative fluorescence units in either channel, resulting in excellent signal-to-noise ratios as high as 11-fold. The transcript-abundance profiles obtained from the two sets of arrays were similar as well (Figure 4). The correlation between the two sets of Cy<sup>3</sup>-cDNA and Cy<sup>5</sup>-cDNA data was 98% and 96%, respectively.

## Conclusions

With the introduction of the Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems, microarray customers now have the flexibility to choose an integrated start-to-finish system that provides their preferred method of generating labeled cDNA. With either the ChipShot<sup>TM</sup> Direct Labeling or the ChipShot<sup>TM</sup> Indirect Labeling Systems, only 5µg total RNA template is required for efficient cDNA synthesis, and one labeling reaction provides sufficient labeled cDNA for multiple full-sized slide hybridizations. The new ChipShot<sup>TM</sup> Membrane Clean-Up System provides a fast, simple technique for efficient recovery of highly purified cDNA.

Both the Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems demonstrate high levels of consistency and reproducibility in array hybridizations. Additionally, good correlation is observed when comparing hybridization data generated using the Pronto!<sup>TM</sup> Plus Direct and Pronto!<sup>TM</sup> Plus Indirect Systems on both cDNA and oligonucleotide arrays.

## Acknowledgments

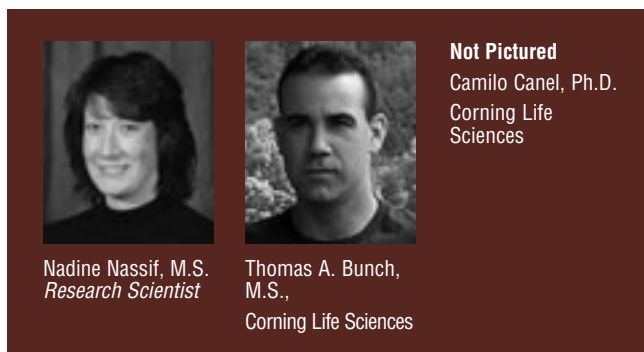
We thank Agilent Technologies for providing the 2100 Bioanalyzer.

## References

1. Brisco, P. *et al.* (1997) *Promega Notes* **64**, 7–12.
2. *Pronto!<sup>TM</sup> Plus Direct Systems Technical Manual #TM243*, Promega Corporation.
3. Skrypina, N.A. *et al.* (2003) *J. Biotechnol.* **105**, 1–9.

## Protocols

- ◆ *Pronto!<sup>TM</sup> Plus Direct Systems Technical Manual #TM243*, Promega Corporation.  
([www.promega.com/tbs/tm243/tm243.html](http://www.promega.com/tbs/tm243/tm243.html))
- ◆ *Pronto!<sup>TM</sup> Plus Indirect Systems Technical Manual #TM261*, Promega Corporation.  
([www.promega.com/tbs/tm261/tm261.html](http://www.promega.com/tbs/tm261/tm261.html))



## Ordering Information

Product	Size	Cat.#
Pronto! <sup>TM</sup> Plus Direct System (with RNA Isolation)	25 reactions	40055
Pronto! <sup>TM</sup> Plus Direct System (without RNA Isolation)	25 reactions	40056
Pronto! <sup>TM</sup> Plus Indirect System (with RNA Isolation)	25 reactions	40075
Pronto! <sup>TM</sup> Plus Indirect System (without RNA Isolation)	25 reactions	40076

<sup>(a)</sup> U.S. Pat. Nos. 6,027,945 and 6,368,800, Australian Pat. No. 732756 and other patents and patents pending.

<sup>(b)</sup> Australian Pat. No. 730718 and other patents and patents pending.

<sup>(c)</sup> U.S. Pat. No. 6,218,531, Australian Pat. No. 745185 and other patents pending.

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